



**PHD**

**Interleukin-3 (IL-3) signal transduction in haemopoietic cells: The role of SHP-1, SHP-2, and SHC**

Bone, Heather Karyn

*Award date:*  
1999

*Awarding institution:*  
University of Bath

[Link to publication](#)

**Alternative formats**

If you require this document in an alternative format, please contact:  
[openaccess@bath.ac.uk](mailto:openaccess@bath.ac.uk)

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

**Take down policy**

If you consider content within Bath's Research Portal to be in breach of UK law, please contact: [openaccess@bath.ac.uk](mailto:openaccess@bath.ac.uk) with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

**INTERLEUKIN-3 (IL-3) SIGNAL TRANSDUCTION IN HAEMOPOIETIC  
CELLS: THE ROLE OF SHP-1, SHP-2, AND SHC**

Submitted by  
Heather Karyn Bone

for the degree of PhD  
of the University of Bath

1999

**Copyright**

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purpose of consultation.

*Heather Bone*

UMI Number: U601551

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U601551

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

UNIVERSITY OF DATH		
LIBRARY		
40	25 NOV 1980	
PHD		



## SUMMARY

Interleukin-3 (IL-3) is a pleiotropic cytokine which binds to a heterodimeric receptor composed of a cytokine-specific  $\alpha$  chain and a  $\beta$  chain, common to the IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) receptors. Although both receptor chains lack intrinsic tyrosine kinase activity, stimulation of haemopoietic cells with IL-3 induces tyrosine phosphorylation of a number of cellular protein substrates including the IL-3 receptor  $\beta$  subunit itself, the protein tyrosine phosphatases (PTPases) SHP-1 and SHP-2, the p52 and p46 isoforms of the adaptor protein Shc, the inositol polyphosphate-5-phosphatase SHIP, and the mitogen activated protein (MAP) kinases erk1 and erk2. Using glutathione S transferase (GST) fusion proteins, it was demonstrated that both the PTPases, SHP-1 and SHP-2, associate with the tyrosine phosphorylated  $\beta$  chain of the IL-3 receptor following IL-3 stimulation. This interaction was direct and mediated by the SH2 domains of the PTPases. Phosphopeptide competition analyses, using peptides based on  $\beta$  chain tyrosine residues, identified the major site of interaction at tyrosine 612. Similarly, the interaction of Shc with the IL-3 receptor  $\beta$  chain was also determined to be direct and could be mediated by both the SH2 and PTB domains of Shc. The SH2 domain of Shc also interacted with residues surrounding tyrosine 612 of the  $\beta$  chain, whereas the PTB domain associated with residues surrounding tyrosine 577. Further investigation into IL-3-induced association of Shc with other phosphoproteins revealed that the SH2 domain of Shc also associated with a novel 100 kDa protein. In addition, the PTB domain of Shc interacted with a tyrosine phosphorylated 145 kDa protein which was determined to be SHIP. To investigate the functional importance of these interactions mediated by Shc in regulating IL-3-induced signalling events, expression of various Shc mutants in a murine IL-3-dependent cell line, Ba/F3, and analyses of the functional consequences of their expression was investigated. Clones were generated exhibiting high levels of inducible expression and low basal levels of expression of full length (FL) Shc and the following Shc mutants: the PTB domain alone, the SH2 domain alone; and a variant with tyrosine 317 (the Grb2 SH2 binding site) mutated to phenylalanine. The profile of IL-3-induced tyrosine phosphorylation of cellular substrates, IL-3-induced Erk1 and Erk2 activation and IL-3-induced proliferation were examined. Expression of the individual SH2 or PTB domains had no detectable effect on any IL-3-induced events investigated. Expression of FL or Y317F resulted in a consistent decrease in endogenous Shc phosphorylation. Erk activation was enhanced in cells expressing FL Shc and reduced in cells expressing the Y317F mutant. However, little effect on IL-3-induced proliferation was observed in cells expressing these Shc variants.

## **Acknowledgements**

I would like to take this opportunity to thank the many family, friends, and colleagues who offered their support and encouragement throughout my studies. First, I would like to thank my Mom, Judy, my Dad, Mike, and my brother, David, for their initial support in my decision to move to the U.K. and for their continued love and encouragement. I would also like to thank my supervisor, and friend, Dr. Melanie Welham, for her encouragement and motivation. Who would have believed you could move me to the U.K. and then get me to stay on and do a PhD. It wasn't all that bad ... really. And then there's the "2.29 Gang" (Pete, Al, and Bev) who welcomed me to the lab with open arms - and a pint in each hand. Thanks for the driving lesson. In addition, I would also like to thank my colleagues and friends Bridget, Helen, Heather, Karen and Ellie, who were always there for a laugh or a gossip and were also there for advice and support during the hard times. Finally, I'd like to thank Tim for his love, understanding and patience. I wouldn't have made it through without you all.

## **Contents**

<b>Title page</b>	<b>i</b>
<b>Summary</b>	<b>ii</b>
<b>Acknowledgements</b>	<b>iii</b>
<b>Contents</b>	<b>iv</b>
<b>List of Figures</b>	<b>ix</b>
<b>List of Tables</b>	<b>xi</b>
<b>List of Abbreviations</b>	<b>xiii</b>
<b>CHAPTER I. Introduction</b>	<b>1</b>
I.A Haemopoiesis	2
I.B. The cytokine super family	3
I.C Interleukin 3 (IL-3)	5
I.C.1 Identification	5
I.C.2 Structure	5
I.C.3 Physiological role	6
I.D Cytokine Receptors	8
I.D.1 The protein tyrosine kinase receptor family	8
I.D.2 Class I cytokine receptors or the Cytokine receptor superfamily	9
I.D.3 Class II cytokine receptors	10
I.E The IL-3 receptor	10
I.F SH2, SH3 and PTB domains	14
I.F.1 SH2 domains	14
I.F.2 SH3 domains	17
I.F.3 PTB domains	19
I.G Proteins involved in IL-3 signalling	20
I.G.1 Protein tyrosine phosphatases (PTPases)	20

I.G.1a SHP-1	21
I.G.1b SHP-2	23
I.G.2 Shc	26
I.G.3 SHIP	28
I.H Signalling pathways activated by IL-3	30
I.H.1 The Ras/MAP kinase pathway	30
I.H.1a Ras	32
I.H.1b Son of Sevenless (Sos)	33
I.H.1c Growth factor receptor-bound protein 2 (Grb2)	35
I.H.1d Protein-protein interactions controlling Ras activation	36
I.H.1e Raf-1	38
I.H.1f MEK	41
I.H.1g Extracellular-signal-regulated kinases (Erks)	42
I.H.2 Phosphoinositide 3-kinase (PI3-K)	44
I.H.3 The Jak-STAT pathway	46
I.H.3a The Janus kinase (Jak) family	46
I.H.3b Signal Transducers and Activators of Transcription (STATs)	49
I.I Overview of IL-3 signal transduction	51
I.I.1 General	51
I.I.2 Involvement of SHP-1 and SHP-2 in IL-3 signalling	51
I.I.3 Involvement of Shc in IL-3 signalling	53
I.J Rationale and Specific Aims	54
<b>CHAPTER II. Materials and Methods</b>	<b>56</b>
II.A. Molecular Biology Techniques	57
II.A.1. Phenol/Chloroform extractions	57
II.A.2. Ethanol precipitations	57
II.A.3. Preparation of competent <i>E.coli</i>	57
II.A.4. Transformation of <i>E.coli</i>	58
II.A.5. Small scale plasmid preparation	58
II.A.6. Large scale plasmid preparation	59
II.A.7. Restriction enzyme digestion	59
II.A.8. Conversion of 5' protruding ends to blunt ends	59

II.A.9. Treatment of plasmid DNA with Calf Intestinal Phosphatase (CIP)	60
II.A.10. Ligations	60
II.A.11. Agarose gel electrophoresis	60
II.A.12. Gel purification of DNA fragments	61
II.A.13. Polymerase chain reaction	61
II.B. Tissue Culture Techniques	61
II.B.1 Cell culture	61
II.B.2. Preparation of conditioned media	62
II.B.3. Transfections	62
II.B.4. Screening for tetracycline-regulated expression	63
II.B.5. XTT dye reduction proliferation assay	64
II.B.6. Cell storage	64
II.C. Protein Chemistry Techniques	64
II.C.1. Cell stimulation and growth factors	64
II.C.2. Immunoprecipitations	65
II.C.3. Bacterial expression and purification for GST fusion proteins	66
II.C.4. Phosphopeptides	67
II.C.5. Phosphopeptide competition assay	67
II.C.6 SDS polyacrylamide gel electrophoresis	67
II.C.7. Immunoblotting (“Western Blotting”)	68
II.C.8. Stripping of immunoblots	69
II.C.9. Bradford protein estimations	69
II.C.10. Immune complex <i>in vitro</i> MAP kinase assays	69
II.D. Materials	71
II.D.1. Antibodies	71
II.D.2. Phosphopeptides	72
II.D.3. Reagents	72

### **CHAPTER III. Protein-Protein Interactions Mediated by the Protein**

<b>Tyrosine Phosphatases SHP-1 and SHP-2 in response to IL-3</b>	<b>75</b>
III.A. Introduction and Aims	76
III.B. Tyrosine phosphorylated $\beta$ c co-precipitates with SHP-1 and SHP-2 after IL-3 stimulation	76

III.C. SHP-1 and SHP-2 associate with $\beta c$ through their SH2 domains after IL-3 stimulation	80
III.D. Binding of SHP-1 and SHP-2 to $\beta c$ is inhibited by a phosphopeptide based on sequences surrounding tyrosine 612 of $\beta c$	83
III.E. Discussion	88
<b>CHAPTER IV. Protein-Protein Interactions Mediated by Shc in Response to IL-3</b>	96
IV.A. Introduction and Aims	97
IV.B. Preparation of Shc-GST fusion proteins	98
IV.C. Precipitation of tyrosine phosphorylated proteins from IL-3 stimulated cells by Shc-GST fusion proteins	100
IV.D. Interaction of Shc with SHIP	103
IV.E. Interaction of Shc with $\beta c$ /Aic2A	108
IV.E.1. Shc binds $\beta c$ directly through both its PTB and SH2 domains	108
IV.E.2. Binding of the Shc SH2 domain to tyrosine phosphorylated $\beta c$ /Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 612 of $\beta c$ /tyrosine 610 of Aic2A	110
IV.E.3. Binding of the Shc PTB domain to tyrosine phosphorylated $\beta c$ /Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 577 of $\beta c$ / tyrosine 575 of Aic2A	114
IV.E.4. Binding of FL Shc to tyrosine phosphorylated $\beta c$ /Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 577 of $\beta c$ / tyrosine 575 of Aic2A	118
IV.F. Interaction of Shc with a tyrosine phosphorylated 100 kDa protein in Ba/F3 cells	120
IV.G. Discussion	123
<b>CHAPTER V Expression of Shc mutants in Ba/F3 cells</b>	131
V.A. Introduction and Aims	132

V.B. The tetracycline-regulated expression system	132
V.C. Construction of pUHD10-3neo expression vectors	135
V.D. Expression of Shc mutants in IL-3 dependent Ba/F3 cells	138
V.E. The expressed Shc variants interact with tyrosine phosphorylated proteins in response to IL-3	139
V.F. Effects of expression of Shc variants on IL-3-induced tyrosine	142
V.F.1. Effects on overall IL-3-induced tyrosine phosphorylation	145
V.F.2. Effects of expression of Shc variants on endogenous Shc phosphorylation	147
V.F.3. Effects of expression of Shc variants on SHP-2 tyrosine phosphorylation	153
V.G. Effects of expression of Shc variants on IL-3-induced MAP kinase activation	156
V.G.1. Expression of FL Shc	156
V.G.2. Expression of Y317F Shc	159
V.G.3. The expressed Y317F Shc mutant does not interact well with the SH2 domain of Grb2	162
V.G.4. Expression of the SH2 domain alone	164
V.G.5. Expression of the PTB domain alone	167
V.H. Effects of expression of Shc variants on proliferation in response to IL-3	170
V.I. Discussion	174
V.I.1 Endogenous Shc phosphorylation is reduced upon FL and Y317F Shc expression	175
V.I.2 Involvement of Shc in IL-3-mediated activation of the Ras/MAP kinase pathway	176
V.I.3 Requirement of Shc for IL-3-dependent proliferation	181
V.I.4 General Observations	182
<b>CHAPTER VI General Discussion</b>	<b>184</b>
<b>APPENDIX I Statistical Analysis</b>	<b>189</b>
<b>References</b>	<b>193</b>
<b>List of Publications</b>	<b>220</b>

## List of Figures

### CHAPTER I

Figure I.1 Hierarchy of haemopoietic stem and progenitor cells	4
Figure I.2 The human high affinity IL-3 receptor	11
Figure I.3 Relationship between IL-3, GM-CSF, and IL-5 receptors in the human and mouse	12
Figure I.4 Selected proteins with SH2, PTB and SH3 domains	15
Figure I.5 SH2 domain binding pockets	18
Figure I.6 Schematic representation of a subgroup of intracellular PTPases containing two SH2 domains amino-terminal to their phosphatase domain	21
Figure I.7 The different isoforms of Shc	27
Figure I.8 Schematic representation of the various domains of SHIP	29
Figure I.9 Mitogen-activated protein kinase cascades	31
Figure I.10 Cycling of p21 <sup>ras</sup> between GTP and GDP bound states	34
Figure I.11 Association of Grb2-Sos complexes with activated receptors	37
Figure I.12 Schematic representation of Raf-1	39
Figure I.13 Structure of Jaks and STATs	47
Figure I.14 Model of IL-3 signal transduction	52

### CHAPTER III

Figure III.1 SHP-1 and SHP-2 associate with tyrosine phosphorylated $\beta c$ after IL-3 stimulation	78
Figure III.2 Schematic representation of the SH2 domains of SHP-1 and SHP-2 expressed as GST fusion proteins	81
Figure III.3. SHP-1 and SHP-2 directly associate with tyrosine phosphorylated $\beta c$ through their SH2 domains	82
Figure III.4. Phosphopeptide pY612 inhibits binding of SHP-1 and SHP-2 to $\beta c$	85
Figure III.5. Model: the role of SHP-1 and SHP-2 in mediating IL-3 signalling Pathways	94

### CHAPTER IV

Figure IV.1 Oligonucleotides used for the cloning of various domains of Shc	99
Figure IV.2 Schematic representation of p52 <sup>Shc</sup> domains expressed as GST fusion proteins	100



Figure IV.3 Shc-GST precipitations from TF-1 and Ba/F3 cells	101
Figure IV.4 SHIP is detected in TF-1 and Ba/F3 cells	104
Figure IV.5 SHIP interacts with the PTB domain of Shc	105
Figure IV.6 The PTB domain of Shc directly interacts with SHIP	107
Figure IV.7 Shc directly associates with tyrosine phosphorylated $\beta$ c through both its SH2 and PTB domains	109
Figure IV.8 Phosphopeptide pY612 inhibits binding of the SH2 domain of Shc to tyrosine phosphorylated $\beta$ c	111
Figure IV.9 Phosphopeptide pY610 inhibits binding of the SH2 domain of Shc to tyrosine phosphorylated Aic2A	113
Figure IV.10 Phosphopeptide pY575 inhibits binding of the PTB domain of Shc to tyrosine phosphorylated $\beta$ c	115
Figure IV.11 Phosphopeptide pY575 inhibits binding of the PTB domain of Shc to tyrosine phosphorylated Aic2A	117
Figure IV.12 Phosphopeptide pY575 inhibits binding of FL Shc to tyrosine phosphorylated $\beta$ c/Aic2A	119
Figure IV.13 The SH2, but not the PTB, domain of Shc binds directly to p100	121
Figure IV.14 Model: the role of Shc in mediating IL-3 signalling pathways	129

## CHAPTER V

Figure V.1. Two vector tetracycline controlled regulatory gene expression system	134
Figure V.2. Cloning steps involved in construction of Shc pUHD10-3neo expression vectors	136
Figure V.3. Expression of myc-tagged Shc proteins	140
Figure V.4. Association of the expressed Shc variants with tyrosine phosphorylated proteins	143
Figure V.5. Effects on IL-3-induced tyrosine phosphorylation in whole cell extracts from cells expressing Shc variants	146
Figure V.6. IL-3-induced endogenous Shc phosphorylation in cells expressing Shc variants	148
Figure V.7. IL-3-induced endogenous Shc phosphorylation is decreased in cells expressing FL and Y317F Shc but not the SH2 domain alone	151
Figure V.8. IL-3-induced SHP-2 phosphorylation is not significantly affected in cells expressing Shc variants	154
Figure V.9. Expression of FL Shc does not have a significant effect on IL-3-	

induced MAP kinase activation in <i>in vitro</i> kinase assays	157
Figure V.10. Activation of erk1 and erk2 in response to IL-3 is moderately accelerated in FL Shc expressing cells	158
Figure V.11. Expression of Y317F Shc does not have a significant effect on IL-3-induced MAP kinase activation observed in <i>in vitro</i> kinase assays	160
Figure V.12. Activation of erk1 and erk2 in response to IL-3 is decreased in Y317F Shc expressing cells	161
Figure V.13. Interaction of the expressed FL and Y317F Shc proteins with the SH2 domain of Grb2	163
Figure V.14. Expression of the SH2 domain alone does not have a significant effect on IL-3-induced MAP kinase activation in <i>in vitro</i> kinase assays	165
Figure V.15. Activation of erk1 and erk2 in response to IL-3 is not affected in cells expressing the SH2 domain alone	166
Figure V.16. Expression of the PTB domain alone does not have a significant effect on IL-3-induced MAP kinase activation in <i>in vitro</i> kinase assays	168
Figure V.17. Activation of erk1 and erk2 in response to IL-3 is not affected in cells expressing the PTB domain of Shc alone	169
Figure V.18. Expression of the Shc variants has no effect on IL-3 responsiveness in XTT dye reduction assays	171

## List of Tables

### CHAPTER I

Table I.1 Recognition specificities of SH2 domains	16
--	----

### CHAPTER II

Table II.1. Antibodies used for blotting and precipitation	71
Table II.2. Synthetic phosphopeptides corresponding to tyrosine residues within $\beta c$ and Aic2A	72
Table II.3. List of Reagents	72

### CHAPTER V

Table V.1. Plasmids used for construction of pUHD10-3neo expression vectors	137
Table V.2. Summary of Shc variants expressed from pUHD10-3neo	137

## APPENDIX I

Table VII.1. Volumes and calculated ratios of endogenous p52 <sup>Shc</sup> tyrosine phosphorylated bands in whole cell extracts	190
Table VII.2. Volumes and calculated ratios of endogenous p52 <sup>Shc</sup> tyrosine Phosphorylated bands precipitated by Grb2SH2-GST	191
Table VII.3. Results of One Sample t-test	191

## **List of Abbreviations**

<b>Aic2A</b>	murine IL-3 receptor $\beta$ subunit
<b>BSA</b>	bovine serum albumin
<b>BCR</b>	B-cell antigen receptor
<b>CAM</b>	cell-surface adhesion molecule
<b>CFU</b>	colony-forming unit
<b>CH</b>	collagen homology (domain)
<b>CNTF</b>	ciliary neurotrophic factor
<b>CSF</b>	colony-stimulating factor
<b>DMSO</b>	dimethyl sulfoxide
<b>DNA</b>	deoxyribonucleic acid
<b>EDTA</b>	ethylenediamine-tetraacetic acid disodium salt
<b>EGF</b>	epidermal growth factor
<b>Epo</b>	erythropoietin
<b>erk</b>	extracellular-signal-regulated kinase
<b>FGF</b>	fibroblast growth factor
<b>FL Shc</b>	full length Shc
<b>GAP</b>	GTPase activating protein
<b>GAS</b>	gamma interferon activated site
<b>G-CSF</b>	granulocyte colony stimulating factor
<b>GDP</b>	guanosine diphosphate
<b>GM-CSF</b>	granulocyte macrophage colony stimulating factor
<b>GNEF</b>	guanine nucleotide exchange factor
<b>Grb2</b>	growth factor receptor-bound protein 2
<b>GST</b>	glutathione S transferase
<b>GTP</b>	guanosine triphosphate
<b>GTPase</b>	guanosine triphosphatase
<b>IFN</b>	interferon
<b>IL</b>	interleukin
<b>IPTG</b>	isopropyl $\beta$ -thiogalactopyranoside
<b>IRS-1/-2</b>	insulin receptor substrate -1/-2
<b>Jak</b>	Janus kinase
<b>JNK</b>	c-Jun amino-terminal kinase

<b>KIR</b>	natural killer cell inhibitory receptor
<b>Ksr</b>	kinase suppressor of Ras
<b>MAP kinase</b>	mitogen activated protein kinase
<b>MBP</b>	myelin basic protein
<b>M-CSF</b>	macrophage colony stimulating factor (also known as CSF-1)
<b>MEK</b>	MAPK/ERK kinase
<b>MP-1</b>	MEK partner 1
<b>mT</b>	middle-T
<b>NAD</b>	nicotinamide adenine dinucleotide
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate
<b>NF-1</b>	neurofibromin
<b>NGF</b>	nerve growth factor
<b>NP-40</b>	Nonidet P-40
<b>OD</b>	optical density
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PBS</b>	phosphate buffered saline
<b>PCR</b>	polymerase chain reaction
<b>PDGF</b>	platelet derived growth factor
<b>PH</b>	pleckstrin homology (domain)
<b>PI3-K</b>	phosphoinositide 3'-kinase
<b>PLC</b>	phospholipase C
<b>cPLA2</b>	cytoplasmic phospholipase A2
<b>PMS</b>	phenazine methosulphate
<b>PMSF</b>	phenyl methyl sulfonyl flouride
<b>PP-1</b>	protein phosphatase 1
<b>PRS</b>	proline-rich sequence
<b>PTB</b>	phosphotyrosine binding (domain)
<b>PTB Shc</b>	PTB domain of Shc alone
<b>PTK</b>	protein tyrosine kinase
<b>PTPase</b>	protein tyrosine phosphatase
<b>RNA</b>	ribonucleic acid
<b>rpm</b>	revolutions per minute
<b>Rsk</b>	ribosomal S6 kinase
<b>RTK</b>	receptor tyrosine kinase
<b>SAPK</b>	stress-activated protein kinase

<b>SDS</b>	sodium dodecyl sulphate
<b>SH2</b>	src homology 2 (domain)
<b>SH2 Shc</b>	SH2 domain of Shc alone
<b>Shc</b>	src homology and collagen-like
<b>SHIP</b>	SH2 domain-containing inositol phosphatase
<b>SHP-1</b>	SH2-containing phosphatase 1
<b>SHP-2</b>	SH2-containing phosphatase 2
<b>SIRP</b>	signal-inhibitory regulatory proteins
<b>SLF</b>	Steel factor (or stem cell factor)
<b>Sos</b>	Son of sevenless
<b>SRE</b>	serum response element
<b>SRF</b>	serum response factor
<b>STAT</b>	signal trasducer and activator of transcription
<b>TBS</b>	tris-buffered saline
<b>TE</b>	Tris/EDTA
<b>TEMED</b>	N,N,N',N'-tetramethylethylenediamine
<b>TNF</b>	tumour necrosis factor
<b>Tris</b>	Tris(hydroxymethyl) methylamine
<b>tTA</b>	tetracycline transactivator
<b>UV</b>	ultra-violet
<b>XTT</b>	sodium 3'-[1-[(phenylamine)-carbonyl]-3,4-tetrazolium]- <i>bis</i> (4-methoxy-6-nitro)benzene-sulphonic acid hydrate
<b>Y317F Shc</b>	full length Shc with a tyrosine to phenylalanine mutation at position 317

**CHAPTER I**  
**Introduction**

## **I.A Haemopoiesis**

In adult life, all circulating blood cells originate from a common pool of pluripotent haemopoietic stem cells in the bone marrow (Metcalf, 1989). This stem cell gives rise, after a number of cell divisions and differentiation steps, to a series of myeloid and erythroid progenitor cells, as well as to a common lymphoid stem cell. The earliest detectable myeloid precursor gives rise to granulocytes, erythroid, monocytes and megakaryocytes and is termed CFU<sub>GEMM</sub> (CFU= colony-forming unit in agar culture medium; GEMM= granulocyte, erythroid, monocyte, macrophage). More mature and specialised progenitors are termed CFU<sub>GM</sub> (granulocytes and monocytes), CFU<sub>Eo</sub> (eosinophils), CFU<sub>E</sub> (erythroid), CFU<sub>Baso</sub> (basophils) and CFU<sub>Meg</sub> (megakaryocytes). BFU<sub>E</sub> (burst-forming unit, erythroid) refers to an earlier erythroid progenitor than the CFU<sub>E</sub> (see Fig. I.1).

The stem cell has the capacity for self-renewal, so that, although the marrow is a major site of new cell production, its overall cellularity remains constant in a normal healthy steady state. The progenitor cells are, however, capable of responding to haemopoietic growth factors with increased production of one or other cell line when the need arises. This complex process of haemopoiesis needs to be tightly regulated in order to maintain steady state conditions in health and to meet the requirements for increased and rapid production in infectious states or after blood loss.

The process that maintains steady state levels of blood cells under the influence of the haemopoietic inductive microenvironment is termed constitutive haemopoiesis and is regulated by growth factors as well as cell-to-cell interaction (Miyajima *et al.*, 1988; Arai *et al.*, 1990). Specialised stromal cells (macrophages, fibroblasts, endothelial cells, fat cells and reticular cells) are embedded in an extracellular matrix of collagen containing adhesive proteins (laminin, haemonectin and fibronectin) and proteoglycans. The stem cells are immobilised in the extracellular matrix by their cell surface adhesion molecules (CAMs) and receptors for the attachment peptide arginine-glycine-aspartic acid (RGD) expressed on the adhesive proteins. As the stem cells differentiate, they lose some of these CAMs and receptors for RGD; these changes may be important in allowing the cells to leave the marrow and enter the circulation. In addition, growth factors, produced locally by stromal cells, bind to the extracellular matrix and are presented to immobilised stem cells. The direction in which stem and progenitor cells differentiate depends largely on the



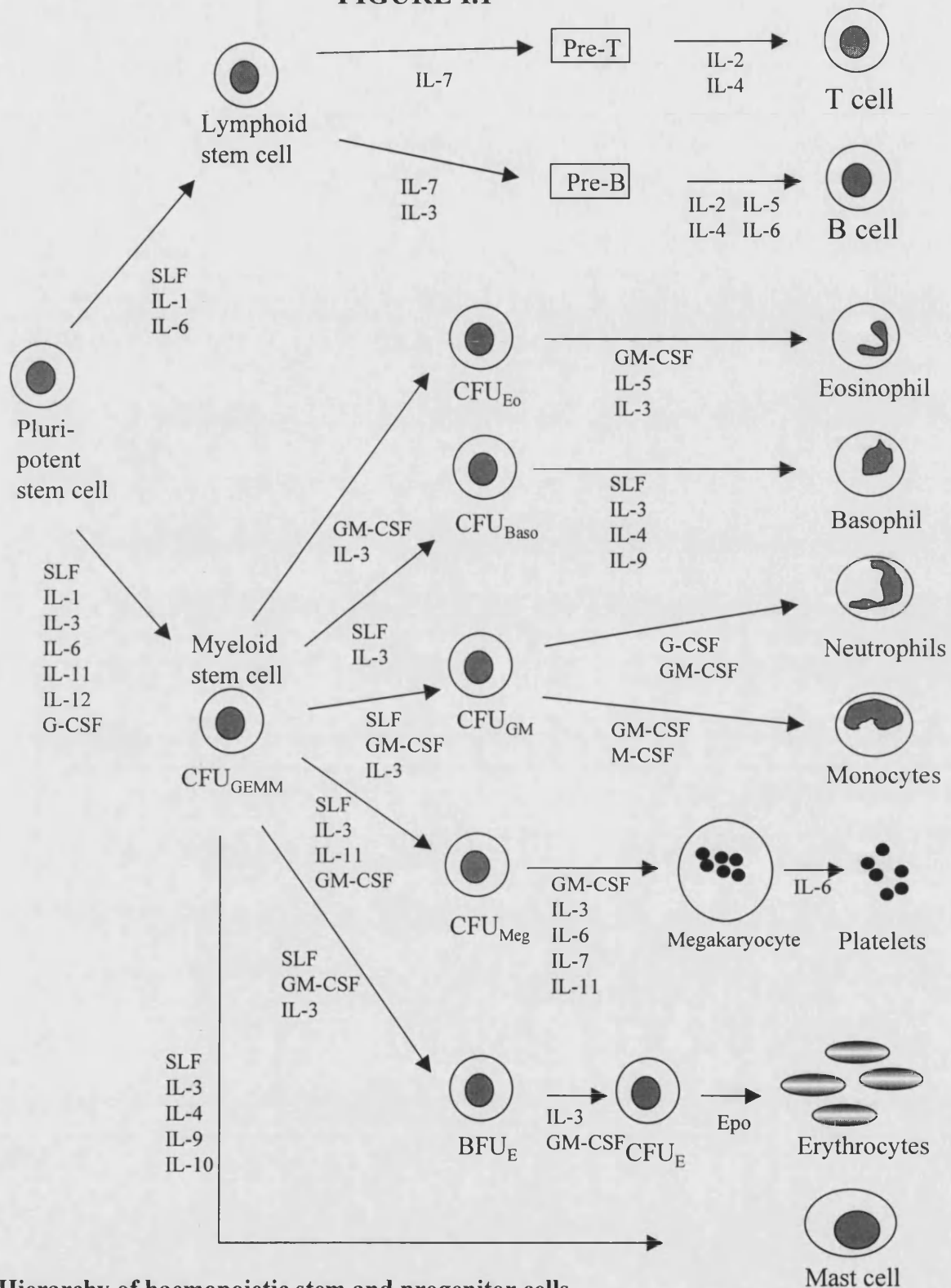
spectrum of growth factors to which they are exposed. This local stromal control seems to maintain stem cell numbers and the production by stem cells of progenitor cells committed to the formation of cells of a particular lineage. The co-ordinated interaction of haemopoietic growth factors further stimulates the proliferation of progenitor cells and their progeny and initiate the maturation events necessary to produce fully mature cells (Metcalf, 1989).

The haemopoietic system must also respond to acute situations such as infection or bleeding. This is accomplished by elaborating growth factors that promote rapid expansion and maturation of specific sets of haemopoietic cells at the affected site. The major source of haemopoietic growth factors that trigger this inducible haemopoiesis are activated T cells and macrophages (Miyajima *et al.*, 1988; Arai *et al.*, 1990). Mast cells, endothelial cells, and fibroblasts also produce overlapping and distinct sets of factors. These growth factors are collectively called cytokines (cyto meaning cell and kine meaning movement). Some of them were discovered because of their ability to stimulate colony-formation in semisolid cultures of bone marrow cells and were therefore termed colony-stimulating factors (CSFs) (Metcalf, 1989). Others were first defined by their actions on lymphocytes and consequently named interleukins (IL) (reviewed by Arai *et al.*, 1990).

### **I.B. The cytokine superfamily**

The cytokine family is now known to consist of a diverse group of acidic glycoproteins with polypeptide molecular masses ranging from 14-39 kDa. There appears to be little amino acid sequence homology between the proteins in this group but analyses of their three- dimensional molecular structures indicate that many exhibit similar structural conformations consisting of four  $\alpha$ -helical bundles, in which two helices combine to produce the active binding domain (Bazan, 1990). To date, multiple cytokines have been identified and include: IL-1 to IL-20, granulocyte macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), macrophage CSF (M-CSF; or CSF-1), *steel* factor (SLF; or stem cell factor, SCF), and erythropoietin (Epo). Extensive research has enabled some of their roles in haemopoiesis to be unravelled and Figure I.1 shows a schematic diagram of haemopoiesis, indicating the different cytokines involved at each stage of development of

FIGURE I.1



### Hierarchy of haemopoietic stem and progenitor cells.

The stem cell gives rise to a common lymphoid stem cell and a myeloid precursor (CFU<sub>GEMM</sub>) which gives rise to more mature and specialised progenitors: CFU<sub>GM</sub> (granulocytes and monocytes), CFU<sub>Eo</sub> (eosinophils), CFU<sub>E</sub> (erythroid), CFU<sub>Baso</sub> (basophils) and CFU<sub>Meg</sub> (megakaryocytes). BFU<sub>E</sub> (burst-forming unit, erythroid) refers to an earlier erythoid progenitor than the CFU<sub>E</sub> (adapted from Miyajima *et al.*, 1992).

the various lineages. However, this model is an oversimplification of haemopoiesis. This is largely because cytokines are generally pleiotropic, i.e., influencing more than one cell type, and many cytokines have overlapping activities (i.e., redundancy). A single cytokine can interact with more than one type of cell through receptors expressed on many cell types. Conversely, the same effect on a particular haemopoietic lineage can be elicited by several cytokines (Arai *et al.*, 1990).

## **I.C Interleukin 3 (IL-3)**

### **I.C.1 Identification**

Interleukin 3 (IL-3) was one of the earliest characterised cytokines because of its profound effects on cells at multiple stages of haemopoietic development (Schrader *et al.*, 1986). IL-3 was therefore discovered independently by a number of laboratories studying different biological activities and went under a variety of names, including: persisting cell stimulating factor (PSF); mast cell growth factor (MCGF); haemopoietic cell growth factor (HCGF); histamine-producing cell-stimulating factor; CFUs stimulating activity; Thy-1-inducing factor; burst promoting activity (BPA), which stimulates the production of erythroid colonies in the presence of erythropoietin; and multi-colony-stimulating factor (multi-CSF), which stimulates multilineage colony formation *in vitro* from bone marrow cells (reviewed by Arai *et al.*, 1990). It was only with biochemical purification (Ihle *et al.*, 1983; Clark-Lewis *et al.*, 1984), molecular cloning and expression (Yokota *et al.*, 1984; Fung *et al.*, 1984) and chemical synthesis (Clarke-Lewis *et al.* 1986) that it was established conclusively that a single protein mediated all of these bioactivities.

### **I.C.2 Structure**

The primary structures for murine (Yokota *et al.*, 1984; Fung *et al.*, 1984), human (Yang *et al.*, 1986), and gibbon (Yang *et al.*, 1986) IL-3 have been deduced from the sequences of cDNA clones. IL-3 has broad structural similarities with other interleukins and haemopoietic growth factors. The murine IL-3 gene encodes a protein of 166 amino acids; the first 26 amino acids encode a typical hydrophobic leader sequence required for secretion. The human IL-3 gene encodes a protein of 152 amino acids, including a leader sequence of 19 amino acids. Consistent with the lack of biological cross-reactivity

between murine and human IL-3, there is little sequence homology between the genes and at the amino acid level, human and murine IL-3 show only 29% homology. The murine gene contains four sites of potential N-glycosylation, while the human gene contains two potential sites. Natural IL-3 occurs in a diversity of glycoforms generated by the addition of carbohydrate groups. Purified, native murine IL-3 exists as a monomer with an apparent molecular size of 28 kDa and contains approximately 38% carbohydrate. Carbohydrate on IL-3 of murine T cell origin is exclusively N-linked (Ziltener *et al.*, 1988). Human IL-3 has an apparent molecular size of 15-30 kDa reflecting heterogeneity in the carbohydrate component. The function of these extensive carbohydrate modifications of the IL-3 polypeptide is unknown. The biological activity of glycosylated IL-3 does not differ *in vivo* when compared with a chemically synthesized, non-glycosylated IL-3, when injected into mice (Ziltener *et al.*, 1994).

### I.C.3 Physiological role

IL-3 has the broadest target specificity of any cytokine. The range of target cells can be summarised as including progenitor cells of every lineage derived from the pluripotential haemopoietic stem cells, with the exception of cells committed to the T and B lymphoid lineages. Thus, IL-3 is capable of stimulating the generation and differentiation of macrophages, megakaryocytes, mast cells, eosinophils, neutrophils, basophils and erythroblasts (summarised by Ihle, 1992).

The question of whether IL-3 has a key role in regulating the production of T or B lymphocytes has been controversial. IL-3 was discovered as a factor in supernatants of activated T lymphocytes that enhances the production of the enzyme 20- $\alpha$ -hydroxysteroid dehydrogenase (20- $\alpha$ -SDH) in spleen cells from athymic (nu/nu) mice (Ihle *et al.*, 1981). 20- $\alpha$ -SDH was thought to be a specific marker of mature T cells and only low levels of enzyme activity are found in the spleens of athymic nude mice. Thus, IL-3 was thought to play a critical role in T-lymphocyte development, inducing differentiation of splenic lymphocytes from nu/nu mice to become 20- $\alpha$ -SDH positive. In fact, the induction of 20- $\alpha$ -SDH formed the basis of the assay for the first purification to homogeneity of IL-3 (Ihle *et al.*, 1983). However, the notion that this enzyme was restricted to T lymphocytes was disproved by the demonstration that IL-3 induced this enzyme in cells of a number of myeloid lineages, including mast cells (Hapel and Young, 1988). Another piece of

evidence in favour of the notion that IL-3 acted on T cells or their precursors was the claim that purified IL-3 promoted the growth of clones of helper T cells which express Thy-1 antigen (Hapel *et al.*, 1981). However, the cells misidentified as helper T lymphocytes were in fact contaminating cells of the myelomonocytic leukaemia WEHI-3B line which had been used as a source for the purification of the IL-3. At the time, the Thy-1 antigen was thought to be a specific marker for T lymphocytes among lymphohaemopoietic cells in the mouse; however, WEHI-3B cells also express Thy-1 antigen. There is some evidence that IL-3 may play a role in the proliferation or differentiation of early lymphoid lineages, largely based on the properties of IL-3-dependent cell lines isolated from foetal liver, bone marrow or spleen. With regard to T lymphocytes, the strongest evidence for an effect of IL-3 has been the identification of IL-3-dependent pro-T cell lines (Sideras and Palacios, 1987). Palacios *et al.* (1984) reported that IL-3 promotes the growth of a population of mouse B lymphocyte precursors, but not of mature B lymphocytes and that IL-3-responsive clones of pre-B lymphocytes could be obtained with high frequency from foetal liver (Palacios *et al.*, 1984). Palacios and Steinmetz (1985) have also reported the generation of a small number of IL-3-dependent cell lines that have the capacity to give rise to B lymphocytes in irradiated animals. Therefore, it appears that IL-3 supports, alone or in combination with other factors, the proliferation of early pluripotent stem cells prior to commitment to the lymphoid lineages. Once committed to the T or B cell lineage, or shortly after commitment, the cells lose the ability to respond to IL-3.

IL-3 may play a more critical role in the development, survival and function of tissue mast cells and blood basophils. These cells are thought to be important effector cells in immunity to parasites and other immunological responses, such as allergic reactions. It has recently been shown using IL-3-deficient mice that IL-3 contributes to the overproduction of mast cells and enhanced basophil development observed in mice infected with the nematode *Stroglyoides venezuelensis* (Lantz *et al.*, 1998). In the IL-3<sup>-/-</sup> mice inoculated with *S. venezuelensis*, a decrease in basophil and mast cells levels, compared to wild type mice, were observed, suggesting that one of the functions of IL-3 in host defence against infection is to expand populations of these haemopoietic effector cells (Lantz *et al.*, 1998).

The major physiological source of IL-3 in both mice and humans is the activated T lymphocyte (Schrader and Nossal, 1980; Schrader, 1981; Niemeyer *et al.*, 1989). It has

also been shown that mast cells can produce IL-3 in response to cross-linking of IgE receptors (Wodnar-Filipowicz *et al.*, 1989; Burd *et al.*, 1989). This has considerable importance since it can be envisioned that with antigenic stimulation, the production of IL-3 could serve to activate or prime other cells in the vicinity of an allergic response, including the mast cells themselves, as well as other haemopoietic cells.

The largely exclusive production of IL-3 by activated T cells has led to the concept that IL-3 may only be involved in immunological regulation of haemopoiesis and serves as a link between the immune system and the haemopoietic system which generates the phagocytic and granulocytic cells that mediate defence and repair. There is little evidence that IL-3 is involved in the steady-state production of blood cells, despite its potent ability to stimulate almost all phases of haemopoiesis. Consistent with this concept, IL-3 is not produced by foetal tissues (Azoulay *et al.*, 1987) or by bone marrow stromal cells under conditions that support haemopoietic stem cell differentiation (Naperstek *et al.*, 1986; Gualtieri *et al.*, 1987). In addition, IL-3 is absent in the serum of normal animals (Crapper *et al.*, 1984). Thus, IL-3 appears to be primarily important for the expansion of haemopoietic cells in an inflammatory response.

### **I.D Cytokine Receptors**

Cytokines exert their biological functions through specific receptors expressed on the cell surface of target cells. Cloning of the individual receptors have revealed their multicomponent nature and shown that different receptors share individual receptor components, providing a potential explanation for some of the remarkable functional pleiotropy and redundancy observed among the activity of several of the cytokines.

#### **I.D.1 The protein tyrosine kinase receptor family**

The receptor tyrosine kinases (RTKs) are a family of more than 50 different transmembrane polypeptides with a protein tyrosine kinase domain in their intracellular portion. Receptors for growth factors such as: epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and Steel factor (SLF) are all members of this family. RTKs, with the exception of the insulin receptor, are all composed of a single polypeptide chain and all share common structural and functional features: a

large extracellular ligand-binding domain, a single membrane spanning domain and a large cytoplasmic domain with tyrosine kinase activity (Ullrich and Schlessinger 1990).

### **I.D.2 Class I cytokine receptors or the Cytokine receptor superfamily**

Unlike the receptor protein tyrosine kinases, receptors of the cytokine receptor superfamily do not have kinase domains, and only a limited similarity is found in their cytoplasmic domains. The majority of cytokines regulating the immune and haemopoietic systems associate with class I cytokine receptors. These receptors include IL-2R ( $\beta$ -chain), and the IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, erythropoietin (Epo), G-CSF, GM-CSF, prolactin, and growth hormone receptors. The high affinity receptors for IL-3, IL-5, GM-CSF, and IL-6 are composed of two subunits, both of which are members of this receptor family. The receptors for IL-3, IL-5 and GM-CSF are composed of a cytokine-specific  $\alpha$ -chain and a signal transducing component shared between the receptors,  $\beta_c$ . The signal transducing component shared between the receptors for IL-6 and IL-11 is gp130. Members of the cytokine receptor superfamily contain a conserved domain of around 200 amino acid residues in their extracellular domains, as well as two or three short conserved motifs in the cytoplasmic region. The conserved 200 amino acid extracellular domain is composed of two fibronectin type III modules, each of which consists of seven  $\beta$ -strands positioned anti-parallel so as to form a barrel-like shape (Bazan, 1990). A trough formed between two barrel-like modules is believed to function as a ligand binding pocket and is where the carboxy-terminal WSXWS motif is located. In addition, four positionally conserved cysteine residues are located in the amino-terminal part of the extracellular domain (reviewed by Bagley *et al.*, 1997). The cytoplasmic domains of these receptors do not contain a consensus catalytic domain and only limited sequence similarity is found. However, two motifs, box 1 and box 2, are relatively well conserved in the cytoplasmic membrane-proximal region of most receptors of this family (Murakami *et al.*, 1991). This region has been shown to be required for mitogenic activity of the growth hormone receptor (Colosi *et al.*, 1993), the GM-CSFR (Ziegler *et al.*, 1993) and the EpoR (Miura *et al.*, 1993). Box 1 comprises a Pro-X-Pro sequence and a preceding cluster of hydrophobic amino acids. Box 2 is only conserved in about 50% of the members of this family: it begins with a cluster of hydrophobic amino acids, followed by negatively charged residues

and ends with one or two positively charged amino acids, which shares limited similarity among most cytokine receptors.

Despite the absence of kinase domains in their receptors, cytokines that utilise receptors of the cytokine receptor superfamily rapidly induce tyrosine phosphorylation of cellular substrate proteins as well as of the receptors. Mutagenesis of several of these receptors has demonstrated that the ability of the receptor to couple ligand binding to protein tyrosine phosphorylation requires the membrane proximal cytoplasmic domain (containing the box 1 motifs), which is also required for mitogenesis (Sato *et al.*, 1993; Sakamaki *et al.*, 1992). The rapid induction of tyrosine phosphorylation, the phosphorylation of the receptors and the detection of protein tyrosine kinase activity in receptor immunoprecipitates have all led to the hypothesis that a protein tyrosine kinase physically associates with the receptor and becomes activated following ligand binding.

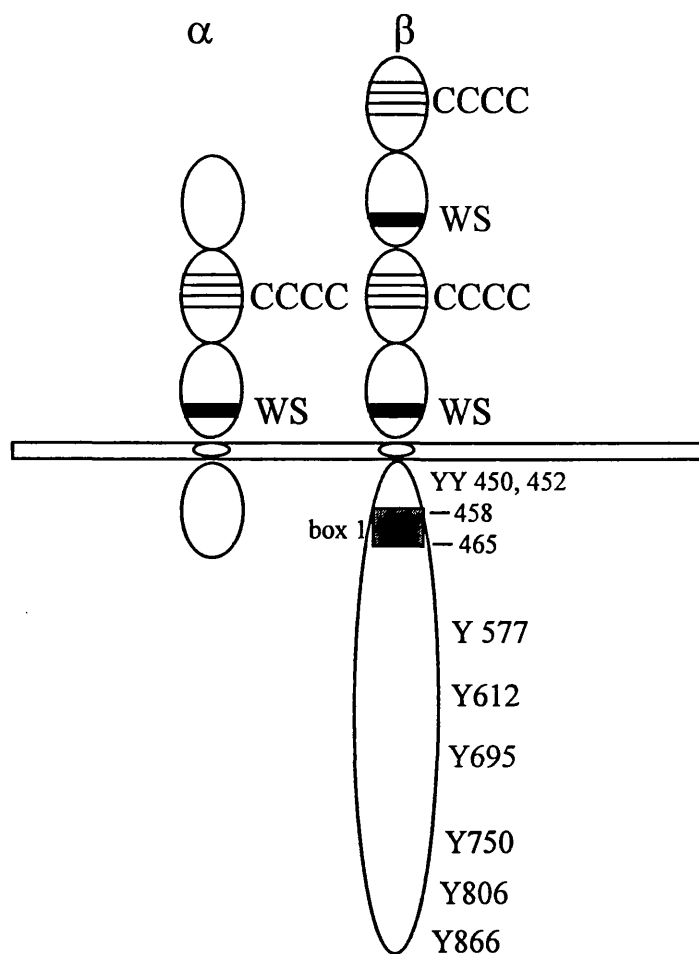
### **I.D.3 Class II cytokine receptors**

The class II cytokine receptor family consists of receptors for interferon (IFN)- $\alpha$ , IFN- $\gamma$  and IL-10. These receptors are multimeric and share overall structural features with the class I cytokine receptors, being distantly related, but more divergent and contain an additional conserved cysteine pair and several conserved prolines and tyrosines.

### **I.E The IL-3 receptor**

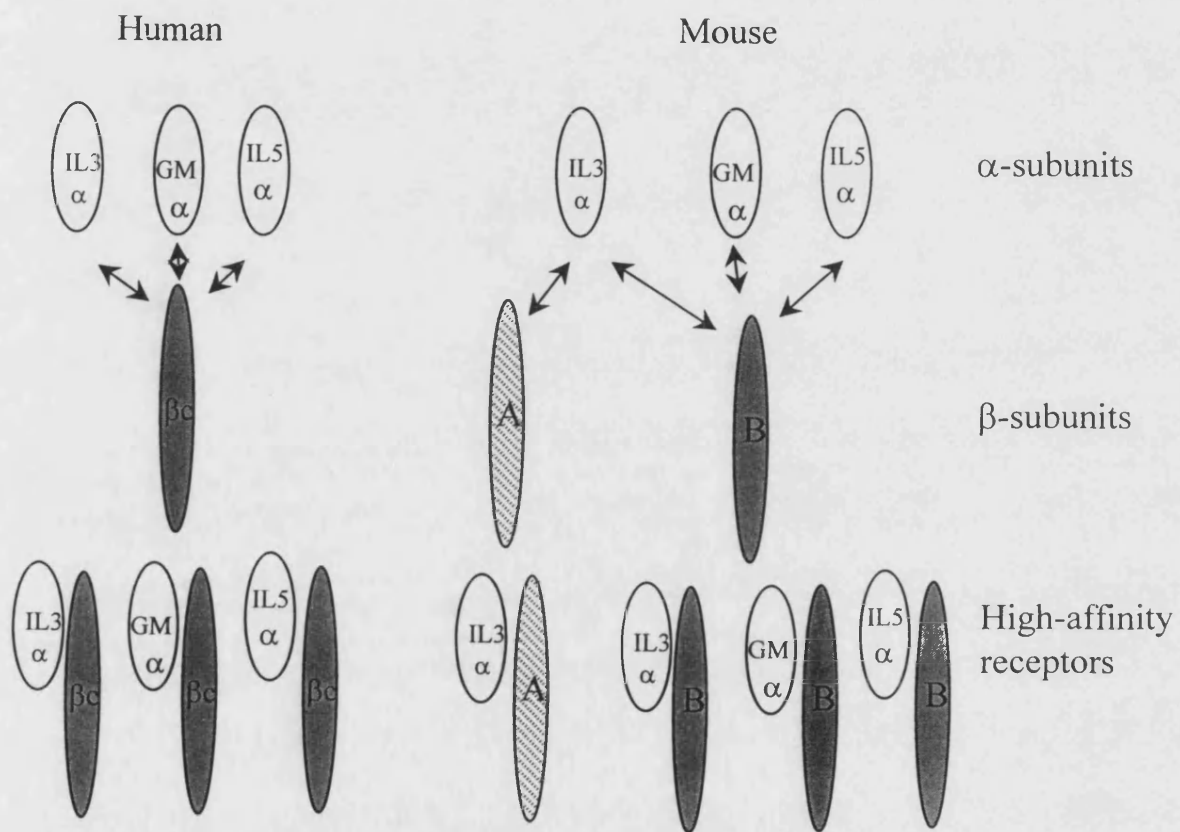
The high affinity receptor for IL-3 is composed of two distinct subunits, the  $\alpha$  and  $\beta$  subunits (see Fig. I.2). Both  $\alpha$  and  $\beta$  subunits belong to the cytokine receptor superfamily and both are required to transduce a signal across the membrane. The  $\alpha$  subunit is a 70 kDa glycoprotein responsible for cytokine-specific binding and alone can bind its ligand with low affinity (Miyajima *et al.*, 1992). The  $\beta$  subunit, a 120-140 kDa glycoprotein, cannot alone bind IL-3 but instead forms a high affinity complex with the  $\alpha$  subunit. The human IL-3R  $\beta$  subunit, called  $\beta_c$ , is shared with the IL-5 and GM-CSF receptors, which each have their own specific  $\alpha$  subunits (IL-5R $\alpha$  and GMR $\alpha$ ) (see Fig. I.3). In mice the situation is complicated by the existence of two different  $\beta$  subunits (Miyajima *et al.*, 1992) (see Fig. I.3). By using an antibody, anti-Aic2, that partially blocks IL-3 binding (Yonehara *et al.*, 1990), a mouse cDNA, Aic2A, was isolated (Itoh *et al.*, 1990). A second



**FIGURE I.2**

### The human high affinity IL-3 receptor.

The extracellular domain of the  $\alpha$  subunit of the IL-3 receptor possesses the conserved motif of the haemopoietic growth factor receptors, containing four conserved cysteine residues (CCCC) and the conserved sequence WSXWS (WS). The extracellular domain of the  $\beta$  subunit has two repeats of this conserved motif. The intracellular domain of the  $\beta$  subunit contains several tyrosine residues and a conserved box 1 domain (shaded region).

**FIGURE 1.3**

### Relationship between IL-3, GM-CSF, and IL-5 receptors in the human and mouse.

Interactions between  $\alpha$  and  $\beta$  subunits are shown as arrows. Aic2A and Aic2B are shown as A and B, respectively.

cDNA, Aic2B, was also isolated that was 91% identical to Aic2A at the amino acid level and had all the common features of the cytokine receptors (Gorman *et al.*, 1990). The human  $\beta_c$  cDNA (KH97) was subsequently isolated by hybridisation using the Aic2A cDNA as a probe (Hayashida *et al.*, 1990). Aic2B is the murine counterpart of the human  $\beta_c$  and with the respective  $\alpha$  chain, can form high affinity receptors for murine IL-3, GM-CSF and IL-5 (Kitamura *et al.*, 1991; Devos *et al.*, 1991). The alternative  $\beta$  subunit, Aic2A, is specific for IL-3 and associates only with IL-3R $\alpha$  to generate a high-affinity IL-3-specific receptor (Itoh *et al.*, 1990). Cloning of the murine IL-3R  $\alpha$  subunit and reconstitution of high affinity IL-3 receptors has showed that the murine IL-3R  $\alpha$  subunit forms high affinity receptors with either Aic2A or Aic2B and no functional difference has been found between these two different forms of the high-affinity murine IL-3 receptor (Hara and Miyajima, 1992).

The  $\beta_c$  subunit, because of its considerably larger cytoplasmic domain, is believed to perform the greater role in signal transduction and is required for a number of intracellular signals, including: tyrosine phosphorylation of a set of cellular proteins, induction of immediate early genes, proliferation and activation of components of the Ras/MAP kinase pathway. Detailed analysis of GM-CSFR  $\beta_c$  (GMR $\beta$ ) deletion mutants have identified at least two distinct functional domains within the cytoplasmic region of GMR $\beta$  that are important for signal transduction (Sato *et al.*, 1993; Sakamaki *et al.*, 1992; Quelle *et al.*, 1994). A membrane proximal region (amino acids 456-517), containing a conserved box 1 (amino acids 458-465) motif, was shown to be essential for proliferation, activation of Jak2 and induction of *c-myc* (Quelle *et al.*, 1994). A second domain (amino acids 627-763) was found to be necessary for activation of Shc, Ras, Raf-1, and MAP kinase as well as induction of *c-fos* and *c-jun* (Sato *et al.*, 1993).

Although neither of the IL-3R subunits possess intrinsic tyrosine kinase activity, one of the earliest events to occur after IL-3 binding to its receptor is induction of protein tyrosine phosphorylation. Many proteins, including the receptor itself (Sakamaki *et al.*, 1992; Duronio *et al.*, 1992a) become tyrosine phosphorylated in response to IL-3, and much effort has been directed towards identifying both these tyrosine phosphorylated substrates and the kinases responsible. The src-like kinases lyn, fyn and hck have been shown to be activated in response to IL-3 (Anderson and Jorgensen, 1995). There is some evidence that fyn and hck associate with  $\beta_c$  (Burton *et al.*, 1997). However, lyn appears to

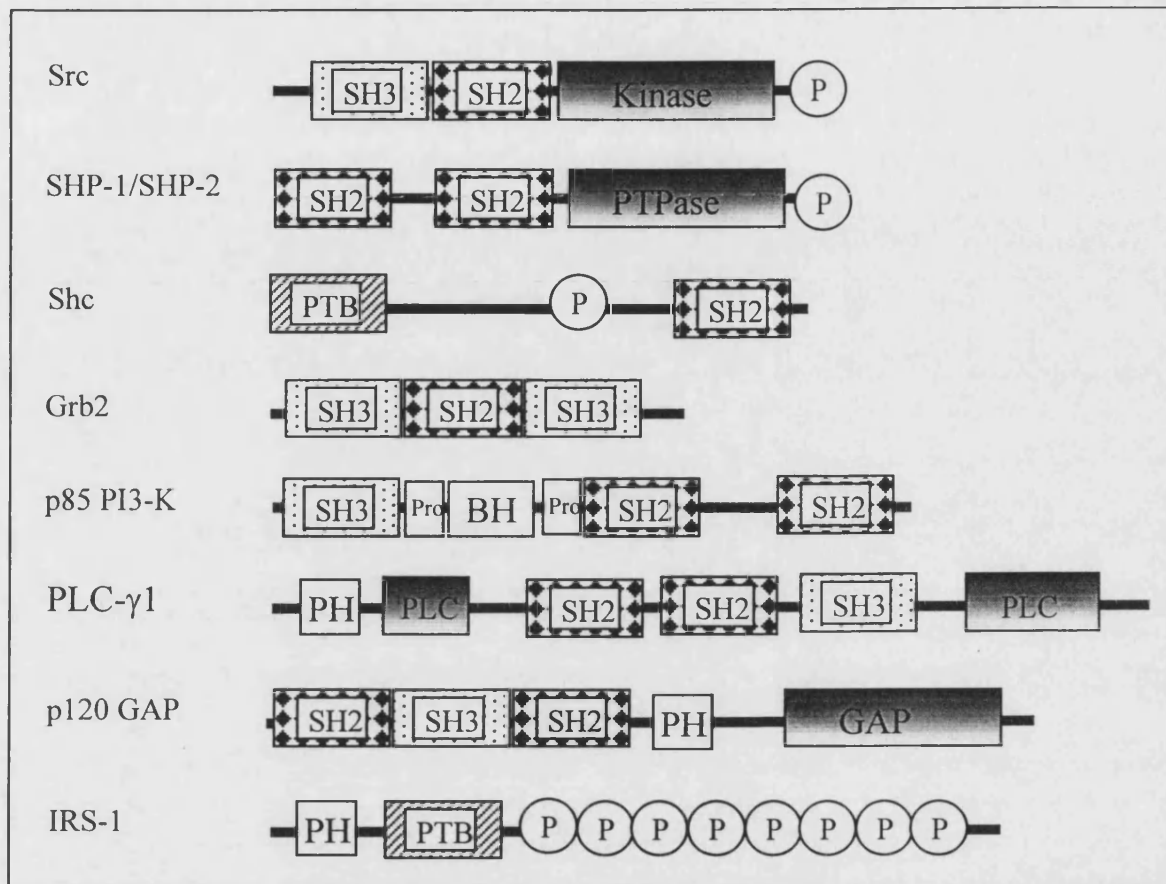
be the prominent src-like kinase associated with  $\beta c$ . A  $\beta c$  receptor truncated at amino acids 517 has been shown to bind lyn (Rao and Mufson, 1995) and recently, lyn has been shown to associate with the membrane proximal region of  $\beta c$  between amino acids 457 and 465, the box 1 domain (Adachi *et al.*, 1999). In addition, a non-Src-like kinase, Jak2, has been shown to be activated by IL-3 (Silvennoinen *et al.*, 1993) and GM-CSF (Quelle *et al.*, 1994) and the box 1 region of  $\beta c$  was shown to be essential for GM-CSF-dependent Jak2 activation (Watanabe *et al.*, 1996). Thus, lyn and Jak2 kinases may be responsible for the IL-3-induced tyrosine phosphorylation of cellular substrates, many of which have been identified and include: p42<sup>erk2</sup> and p44<sup>erk1</sup> (Welham *et al.*, 1992), p120 Jak2 (Silvennoinen *et al.*, 1993), p90 STAT5 (Mui *et al.*, 1995), p70 SHP-2 (Welham *et al.*, 1994b), p46 and p52 Shc (Welham *et al.*, 1994a), and p145 SHIP (Damen *et al.*, 1996). The  $\beta$  subunit of the receptor itself also becomes tyrosine phosphorylated upon IL-3 stimulation (Sakamaki *et al.*, 1992; Duronio *et al.*, 1992a); the 8 potential intracellular tyrosine phosphorylation sites may provide crucial docking sites for signalling molecules containing SH2 or PTB domains.

## **I.F SH2, SH3 and PTB domains**

### **I.F.1 SH2 domains**

SH2 domains are noncatalytic regions of approximately 100 amino acids, originally identified as a non-kinase domain conserved between the *v-fps* and *v-src* cytoplasmic tyrosine kinases; hence the name *src*-homology domain (Sadowski *et al.*, 1986). SH2 domains are found in many cytoplasmic signalling molecules including kinases, phosphatases and adaptor molecules (see Fig. I.4). Those molecules with enzymatic activity which contain SH2 domains include the p60<sup>c-src</sup> family protein tyrosine kinases, PLC- $\gamma$ , the p21<sup>ras</sup> GTPase activating protein, p120<sup>GAP</sup>, and the protein tyrosine phosphatases (PTPases) SHP-1 and SHP-2. Adaptor proteins with no enzymatic activity such as the p85 subunit of phosphoinositol 3' kinase (PI3-K), Shc, Grb2, and IRS-1 also contain SH2 domains (Pawson and Gish, 1992; Pawson, 1995).

SH2 domains have been shown to directly recognise phosphotyrosines embedded within a specific amino acid sequence. Using a degenerate phosphopeptide library, the specificity of individual SH2 domains were determined (Songyang *et al.*, 1993, 1994).

**FIGURE 1.4**

### Selected proteins with SH2, PTB and SH3 domains.

Pro, proline-rich SH3-binding sites; P, phosphotyrosine-containing SH2-binding site; PTPase, phosphotyrosine phosphatase domain; PH, pleckstrin homology domain; BH, bcr homology domain.

Generally, most SH2 domains fall into one of two broad categories. Group I SH2 domains, which include those of the src family kinases, prefer phosphopeptides with the general amino acid motif pY-hydrophilic-hydrophilic-hydrophobic and make specific contacts with the residues immediately following the phosphotyrosine (pY) at the +1 and +3 positions. Group II SH2 domains, including those of phospholipase C - $\gamma$ 1 (PLC- $\gamma$ 1) and SHP-2 protein tyrosine phosphatase, and select phosphopeptides with the general amino acid motif pY-hydrophobic-X-hydrophobic but also make contacts with residues out to the +5 position. The specific motifs recognised by some different SH2 domain containing proteins, as determined by degenerate phosphopeptide libraries (Songyang *et al.*, 1993, 1994), are listed in Table I.1. SHP-1 and SHP-2 both show broad selectivity for pY-hydrophobic-X-hydrophobic amino acid motifs. However, Val and Ile were found to be the preferred hydrophobic residues at the +1 and +3 positions of SHP-2, whereas Phe was slightly preferred by SHP-1 at +1 and +3 (Songyang *et al.*, 1994). In addition, the involvement of residues more carboxy-terminal to these, especially a hydrophobic residue (Leu, Phe, or Pro) at position +5 has also been suggested for high affinity binding in the case of SHP-2 (Huyer *et al.*, 1995). Grb2 is unusual in that it has very weak selectivity at the +3 position and selects primarily on the basis of Asn at +2 (Songyang *et al.*, 1994).

**TABLE I.1**  
**Recognition specificities of SH2 domains**

SH2 domain	Recognition sequence
Src family members	pY-Glu-Glu-Ile
SHP-1	pY-hydrophobic-X-hydrophobic
SHP-2	pY-V/I/T-X-V/L/I-X-L/F/P
Shc	pY-I/E/Y/L-X-I/L/M
p85 N-terminal	pY-X-X-M
Grb2	pY-X-N-X

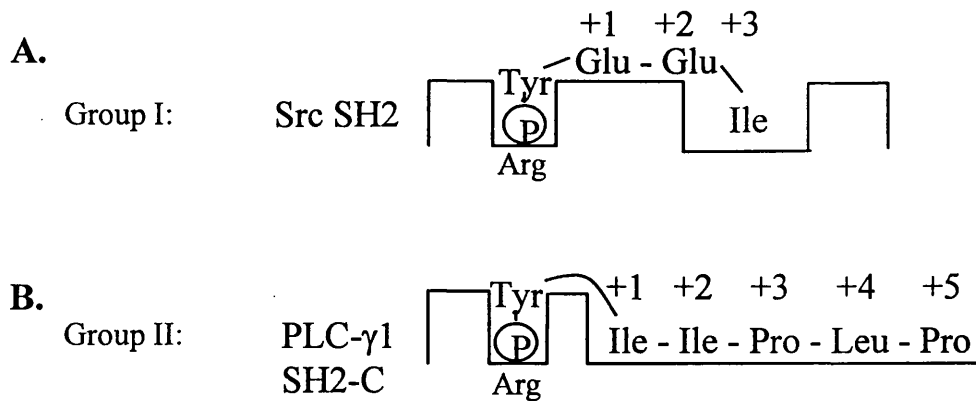
The structure of the SH2 domain, as determined by X-ray crystallographic analysis of the Src SH2 domain complexed with phosphopeptide-containing pentapeptides, is formed from two anti-parallel  $\beta$  sheets, surrounded by two  $\alpha$  helices, forming a two pocket

hydrophobic core (Waksman *et al.*, 1992). The residues conserved between different SH2 domains are involved in forming the hydrophobic core structure and are involved in phosphopeptide recognition. The substrate peptides themselves bind to a fairly flat surface formed by the central  $\beta$  sheet, with the phosphotyrosine protruding into the first pocket which contains an invariant arginine residue (Arg-175, numbered according to v-src) which is located in the FLVERS sequence, the most highly conserved region among all SH2 domains (Koch *et al.*, 1991). This arginine residue is crucial for the ability of SH2 domains to distinguish phosphotyrosine-containing residues from those with phosphoserine or phosphothreonine. Only the phosphotyrosine side chain extends far enough into the pocket to achieve optimal binding with the arginine residue which forms hydrogen bonds with two of the phosphotyrosine phosphate oxygens (Pawson and Gish, 1992). For the group I SH2 domains, the +1 residue makes hydrophobic contacts with the  $\beta$  strand while the second binding pocket encompasses the residue in the +3 position (Waksman *et al.*, 1993) (Fig. I.5 A). This second binding pocket is composed of an invariant tyrosine residue and nonconserved hydrophobic residues, variations of which may regulate the binding at the +3 position and confer specificity of the SH2 domain (Pawson and Gish, 1992). However, the second binding pocket of group II SH2 domains, from proteins such as SHP-2 and PLC- $\gamma$ 1, forms an extended shallow hydrophobic groove structure which makes contacts out to the +5 position of phosphopeptides (Lee *et al.*, 1994; Pascal *et al.*, 1994), (Fig. I.5 B).

Transmission of signals by the binding of SH2 domains to a tyrosine phosphorylated protein occurs via two mechanisms. First, the binding may alter the subcellular localisation of the protein, bringing it closer to its substrate, or closer to a protein that modifies it. Secondly, binding may induce a conformational change which may alter the catalytic activity of the protein (Cohen *et al.*, 1995).

### I.F.2 SH3 domains

SH3 domains are small regions of approximately 55-70 amino acids found in many intracellular signalling proteins including enzymes and adaptors (Koch *et al.*, 1991; Pawson and Gish, 1992; Cantley *et al.*, 1991). These domains are also involved in mediating protein-protein interactions and are frequently present in signalling molecules which also contain SH2 domains as in p60<sup>c-src</sup>, PLC- $\gamma$ , Grb2, p120<sup>GAP</sup> and the p85 subunit

**FIGURE I.5****SH2 domain binding pockets.**

SH2 domains recognise phosphotyrosine (pTyr)-containing amino acid motifs. (A) Group I SH2 domains have a pTyr-binding pocket and a hydrophobic pocket encompassing the residue +3 from the pY. The src SH2 domain is an example of a Group I SH2 domain which binds with high affinity to peptides with the sequence pTyr-Glu-Glu-Ile. (B) The peptide pTyr-Ile-Ile-Pro-Leu-Pro binds to the carboxy-terminal SH2 domain of PLC- $\gamma$ 1, an example of a Group II SH2 domain, which possesses an extended shallow hydrophobic groove which associates with residues extending to the +5 position from the pTyr.



of PI3-K (Pawson and Gish, 1992) (refer to Fig. I.4). SH3 domains recognise short proline rich peptide motifs of approximately 10 amino acids (Ren *et al.*, 1993) composed of a X-P-p-X-P core motif, where X tends to be an aliphatic residue and the two conserved prolines (P) are crucial for high affinity binding. The intervening scaffolding residue (p) also tends to be a proline (Cohen *et al.*, 1995; Pawson, 1995). The interaction of an SH3 domain with its ligand does not depend upon modification such as phosphorylation and are therefore usually constitutive associations. (Pawson, 1995). The structure of SH3 domains is well conserved and consists of five anti-parallel  $\beta$  strands that pack to form two perpendicular  $\beta$  sheets (Cohen *et al.*, 1995). This forms three pockets with which the ligand interacts. Two hydrophobic pockets, sites 1 and 2, are formed by the conserved SH3 aromatic residues and bind each of the X-P pairs. Site 3 frequently binds an arginine residue but is more variable. The specificity of the SH3 domains is conferred by the non-proline residues in the ligand, interacting with two variable loops of the SH3 domain flanking the hydrophobic binding sites (Pawson, 1995). SH3-mediated protein-protein interactions function primarily to localise signalling molecules within the cell (Koch *et al.*, 1991).

### I.F.3 PTB domains

The more recently defined phosphotyrosine-binding (PTB) domain was originally identified as a 186 amino acid amino-terminal segment of the signalling molecule Shc (Kavanaugh and Williams, 1994). This domain bound specifically to an unknown tyrosine phosphorylated protein of 140 kDa but was not structurally similar to members of the SH2 domain family (Kavanaugh and Williams, 1994). Simultaneously, the Shc amino-terminus was also shown to bind specifically with the autophosphorylated EGFR by probing an expression library with the carboxy-terminus of the autophosphorylated EGFR (Blaikie *et al.*, 1994). Using a yeast two hybrid system, a region of IRS-1 that resembles the Shc amino-terminus, in addition to the Shc amino-terminus, were also both shown to bind the autophosphorylation site of the insulin receptor (Gustafson *et al.*, 1995).

PTB domains, like SH2 domains, mediate protein-protein interaction by binding to phosphotyrosine residues; however, unlike the SH2 domain, the specificity of the PTB interaction resides within the amino acids N-terminal to the phosphotyrosine. Expression cloning (Kavanaugh *et al.*, 1995) and phosphopeptide libraries (Songyang *et al.*, 1995) have revealed that PTB domains bind to phosphotyrosines within a sequence motif N-X-X-

pY, where X is any amino acid and pY represents the phosphorylated tyrosine residue. Additionally, the amino acid in the -5 position, relative to the phosphotyrosine, has been reported to be important for PTB domain recognition, with aliphatic residues predominating in this region (Trub *et al.*, 1995; Zhou *et al.*, 1995a). Data derived from examining the binding of the Shc PTB domain to sequence motifs derived from growth factor receptors and oncoproteins has defined the minimal Shc PTB binding motif as N-X-X-pY and suggest a high affinity motif hydrophobic-(D/E)-N-X-X-pY-(W/F) (Lamiet *et al.*, 1996). The nuclear magnetic resonance structure of the Shc PTB domain complexed to a phosphopeptide revealed that the PTB domain forms a complex globular structure consisting of a  $\beta$  sandwich, capped by an  $\alpha$  helix (Zhou *et al.*, 1995a).

## **I.G Proteins involved in IL-3 signalling**

### **I.G.1 Protein tyrosine phosphatases (PTPases)**

In haemopoietic cells, most receptor-mediated signalling pathways involved in physiological processes such as growth, differentiation, metabolism and cell cycle regulation, involve a cascade of protein phosphorylation and dephosphorylation catalysed by kinases and phosphatases (Tonks and Neel, 1996). A proportion of this phosphorylation occurs at tyrosine residues. Initially, protein tyrosine kinases (PTKs) were believed to be the key enzymes controlling phosphorylation *in vivo*, with a small number of protein tyrosine phosphatases (PTPases) playing largely housekeeping roles and down-regulating signalling. However, with the ever increasing number of PTPases being cloned and the subtleties of regulation and diversity of their functions being determined, the importance of PTPases in the regulation of signalling pathways has been heightened. In fact, PTPases not only play negative regulatory roles but also exert positive effects on cellular signalling. Both transmembrane and cytosolic PTPases exist and are defined by a conserved catalytic domain of 240 residues characterised by the unique signature motif [I/V]HCxAGxxR[S/T]G (Barford *et al.*, 1995). A subgroup of intracellular PTPases contain two SH2 domains amino-terminal to their catalytic phosphatase domain (see Fig I.6). Both SHP-1 and SHP-2 are members of this subgroup and have been shown to be important in controlling proliferation and survival in haemopoietic cells.

FIGURE I.6



**Schematic representation of a subgroup of intracellular PTPases containing two SH2 domains amino-terminal to their phosphatase domain.**

### I.G.1a SHP-1

The first intracellular PTPase of this subgroup identified was PTP-1C (Shen *et al.*, 1991), also referred to as HCP (Yi *et al.*, 1992), SH-PTP1 (Plutzkey *et al.*, 1992) and SHP (Matthews, 1992), which is now referred to as SHP-1 for SH2 domain containing phosphatase 1. SHP-1 is a 64 kDa cytoplasmic protein expressed almost exclusively in haemopoietic cells but has also been detected in epithelial cells (Plutzky *et al.*, 1992). Mutations within SHP-1 are responsible for the phenotype of the *motheaten* (*me/me*) or *motheaten* viable (*me<sup>v</sup>/me<sup>v</sup>*) mouse strains (Shultz *et al.*, 1993; Tsui *et al.*, 1993). The *me/me* mutation results from a single nucleotide deletion (at position 228), leading to aberrant splicing and production of an early frameshift; thus no translated SHP-1 protein is produced in *me/me* mice (Tsui *et al.*, 1993). The *me<sup>v</sup>/me<sup>v</sup>* mutation results from a thymidine to adenine transversion, resulting in either a 15 base-pair in-frame deletion or a 69 base-pair in-frame insertion within the sequence encoding the SHP-1 catalytic domain. Thus, the *me<sup>v</sup>/me<sup>v</sup>* mice express two SHP-1 proteins that together retain a small amount (10-20%) of catalytic activity (Tsui *et al.*, 1993). SHP-1 appears to act predominantly as a negative regulator of growth factor signalling as these *motheaten* mice die soon after birth due to the overproliferation and accumulation of macrophages and granulocytes in the lungs (Van Zant and Shultz, 1989). Haemopoietic cells from *motheaten* mice are hyperproliferative in response to Epo (Van Zant and Shultz, 1989), CSF-1 (Chen *et al.*, 1996) and GM-CSF (Jiao *et al.*, 1997). Further evidence for SHP-1's negative regulatory role has been demonstrated in IL-3-dependent cells by examining the effects of increasing and decreasing SHP-1 levels by expressing SHP-1 cDNA in sense and antisense

orientations (Yi *et al.*, 1993). An increase in SHP-1 expression resulted in suppression of IL-3-induced cell growth whereas a slight increase in growth rate resulted from decreased SHP-1 levels (Yi *et al.*, 1993).

SHP-1 has been shown to associate with a number of haemopoietic growth factor receptors including the EpoR (Klingmuller *et al.*, 1995; Yi *et al.*, 1995), *c-kit* (Yi and Ihle, 1993), and the murine IL-3 receptor  $\beta$  subunit, Aic2A (Yi *et al.*, 1993) following activation with their respective ligands. Association occurs via the amino-terminal SH2 domain of SHP-1 which binds to phosphotyrosine sites in the cytoplasmic regions of the receptors. This has been shown to occur via tyrosine 429 of the EpoR (Klingmuller *et al.*, 1995; Yi *et al.*, 1995). However, the site at which SHP-1 interacted with Aic2A was not mapped. In addition, SHP-1 has also been shown to associate with other receptor complexes, such as Fc $\gamma$ RIIB1 (D'Ambrosio *et al.*, 1995), CD22 (Doody *et al.*, 1995), the natural killer cell inhibitory receptor (KIR) (Burshtyn *et al.*, 1996), the B-cell antigen receptor (Pani *et al.*, 1995), the receptor for interferon  $\alpha/\beta$  (David *et al.*, 1995) and a member of the signal-inhibitory regulatory proteins (SIRPs) (Fujioka *et al.*, 1996; Kharitononkov *et al.*, 1997).

Studies using phosphopeptides corresponding to potential phosphorylation sites on the EpoR (Y429) and Aic2A (Y628), have indicated that occupancy of the amino terminal SH2 domain of SHP-1 by these phosphotyrosine residues leads to increases in the catalytic activity of SHP-1 (Pei *et al.*, 1994). Since truncation of SHP-1, either by deleting the SH2 domains or the carboxy-terminus, leads to activation of the PTPase, this suggested that the SH2 domains of SHP-1 interact with the catalytic domain, serving to autoinhibit the phosphatase activity (Pei *et al.*, 1994). However, the two SH2 domains of SHP-1 appear to have different functions. Phosphopeptide analyses in combination with SHP-1 truncation mutants revealed that the amino terminal SH2 domain was necessary and sufficient for autoinhibition and ligand induced activation of SHP-1 while the carboxy-terminal SH2 domain played little role (Pei *et al.*, 1996). Thus, it appears that while the amino-terminal SH2 domain serves both as a regulatory and recruiting domain, the carboxy-terminal SH2 domain acts solely as a localisation domain (Pei *et al.*, 1996).

The mechanism by which SHP-1 negatively regulates signalling pathways has been shown to be via dephosphorylation of key substrates. Recent studies have shown that dephosphorylation of receptor-associated Jak family kinases may be one general mechanism by which SHP-1 negatively regulates signals from cytokine receptors. Cells

expressing mutant Epo receptors which were unable to associate with SHP-1, showed a sustained Jak2 phosphorylation in response to Epo, suggesting that inhibition of SHP-1 binding to the receptor prevented it from dephosphorylating Jak2 (Klingmuller *et al.*, 1995). In addition, in COS-7 cells transfected with Jak2 and SHP-1, a direct association of SHP-1 with Jak2 was demonstrated. This interaction was SH2 domain-independent and lead to SHP-1 activation and dephosphorylation of Jak2 (Jiao *et al.*, 1996). Jak kinases in macrophages from *motheaten* mice, which lack functional SHP-1, were shown to be hyperphosphorylated following  $\alpha/\beta$  interferon-treatment (David *et al.*, 1995) and GM-CSF stimulation has been shown to induce a modest and transient Jak-2 hyperphosphorylation (Jiao *et al.*, 1997). SHP-1 has also been shown to be involved in the dephosphorylation of receptors following activation, such as the murine IL-3 receptor, Aic2A, as increased SHP-1 levels have lead to a reduction in the levels of IL-3-induced tyrosine phosphorylated Aic2A (Yi *et al.*, 1993). In addition, it has also been suggested that SHP-1 dephosphorylates the CSF-1R as the CSF-1R has been shown to become hyperphosphorylated following CSF-1 stimulation of macrophages from *motheaten* mice (Chen *et al.*, 1996). Finally, SHP-1 may also be responsible for the dephosphorylation of additional proteins. This is thought to be mediated through Grb2, which associates via its SH2 domain with SHP-1 following CSF-1 stimulation of normal macrophages. The purpose of this interaction appears to be recruitment of substrates for SHP-1 since a number of proteins which associate with the SH3 domains of Grb2 were found to be hyperphosphorylated in macrophages from *motheaten* mice (Chen *et al.*, 1996).

### I.G.1b SHP-2

Another member of this SH2 domain containing PTPase subgroup is Syp (Feng *et al.*, 1993), also referred to as SH-PTP2 (Freeman *et al.*, 1992), SH-PTP3 (Adachi *et al.*, 1992), PTP-1D (Vogel *et al.*, 1993) and PTP-2C (Ahmad *et al.*, 1993), which is now known as SHP-2. SHP-2 appears to be ubiquitously expressed and is likely to be the mammalian homologue of the *Drosophila* *csw* gene, the gene product of which positively transmits signals downstream of the *torso* receptor protein tyrosine kinase (Perkins *et al.*, 1992). SHP-2 becomes phosphorylated on tyrosine and threonine residues following stimulation with a number of ligands, including those for receptor tyrosine kinases such as EGF and PDGF and *c-kit* (Feng *et al.*, 1993; Vogel *et al.*, 1993; Lechleider *et al.*, 1993a;

Tauchi *et al.*, 1994) and for cytokine receptors such as Epo (Tauchi *et al.*, 1995), IL-3 and GM-CSF (Welham *et al.*, 1994b), INF $\alpha/\beta$  (David *et al.*, 1995), prolactin (Ali *et al.*, 1996) and ciliary neurotrophic factor (CNTF) (Boulton *et al.*, 1994). Although not tyrosine phosphorylated in response to insulin, SHP-2 binds to tyrosine phosphorylated IRS-1 (Kuhne *et al.*, 1993) and acts as a positive mediator of PDGF, insulin and prolactin signals (Bennett *et al.*, 1994; Xiao *et al.*, 1994; Milarski and Saltiel, 1994; Ali *et al.* 1996).

Several reports suggest that SHP-2 is a positive regulator of mitogenic signal transduction pathways. Microinjection of either anti-SHP-2 antibodies, or a SHP-2-SH2-GST fusion protein, into Rat1 fibroblasts overexpressing human insulin receptors blocks insulin-stimulated DNA synthesis (Xiao *et al.*, 1994). The phosphatase activity of SHP-2 has been shown to be critical for SHP-2 to act as a positive regulator. Expression of a catalytically inactive mutant SHP-2 (Cys 459-Ser), or expression of the SHP-2 SH2 domains alone have been shown to lead to a reduction in insulin-stimulated DNA synthesis and MAP kinase activation (Milarski and Saltiel 1994; Noguchi *et al.*, 1994; Yamauchi *et al.*, 1995). In addition, overexpression of catalytically inactive SHP-2 has been shown to inhibit MAP kinase activation following EGF stimulation (Bennett *et al.*, 1996) and SHP-2 phosphatase activity has been shown to be required for fibroblast growth factor (FGF)-induced MAP kinase activation in *Xenopus* embryos (Tang *et al.*, 1995). In addition, SHP-2 has been shown to be required in *Xenopus* embryonic development, as microinjection of catalytically inactive SHP-2 into *Xenopus* embryos blocked FGF mediated mesoderm induction and, in particular, caused severe posterior truncation (Tang *et al.*, 1995). Interestingly, a similar defect in mesodermal patterning was also observed at the early stage of mouse gastrulation in mice homozygous for a SHP-2 mutation containing an internal deletion of 65 amino acids in the amino-terminal SH2 domain (Saxton *et al.*, 1997). These mice die between days 8.5 and 10.5 of gestation with multiple defects in the patterning of the axial mesodermal tissues and posterior development. *Shp-2* null mutant mice also die around the same time of mid-gestation (Arrandale *et al.*, 1996), suggesting that the intact amino-terminal SH2 domain of SHP-2 is required for its physiological function in cells. Using homozygous SHP-2 mutant embryonic stem cells (ES) in *in vitro* ES cell differentiation assays, it was further demonstrated that the deletion mutation in SHP-2 resulted in severe suppression of the development of erythroid progenitors and completely blocked differentiation into myeloid lineages (Qu *et al.*, 1997). Thus, SHP-2 appears to

play a positive role in signalling pathways and to be an essential component for mediating haemopoiesis in mammals.

Many groups have demonstrated the association of SHP-2 with growth factor receptors. The SHP-2 SH2 domains have been shown to associate with the PDGFR, EGFR, *c-kit* (Feng *et al.*, 1993; Vogel *et al.*, 1993; Tauchi *et al.*, 1994) and the EpoR (Tauchi *et al.*, 1995). The amino-terminal SH2 domain of SHP-2, like SHP-1, appears to have a higher affinity for activated receptors than the carboxy-terminal SH2 domain (Lechleider *et al.*, 1993a). Specifically, SHP-2 has been shown to associate with residues surrounding tyrosine 1009 of the PDGFR (Lechleider *et al.*, 1993b; Kazlauskas *et al.*, 1993) and tyrosine 425 of the EpoR (Tauchi *et al.*, 1996).

During its interaction with the activated receptors, SHP-2 is itself tyrosine phosphorylated and its catalytic activity is stimulated. However, it has not been determined whether binding to an activated receptor is a prerequisite for SHP-2 phosphorylation. Studies using phosphotyrosine-containing peptides derived from IRS-1 have indicated that, like SHP-1, occupancy of the SH2 domains by phosphotyrosine residues within a specific motif leads to an increase in the catalytic activity of SHP-2 (Sugimoto *et al.*, 1994; Pluskey *et al.*, 1995). In addition, deletion of the SH2 domains or truncation of the carboxy-terminus has lead to significant increases in phosphatase activity, suggesting an autoinhibitory mechanism similar to that of SHP-1 (Zhao *et al.*, 1994).

Phosphorylation of SHP-2 may be mediated by Jak1 and/or Jak2 as co-transfection studies in COS cells have demonstrated that SHP-2 can associate with Jak1 and Jak2, resulting in phosphorylation of SHP-2 at tyrosine 304 and 327 (Yin *et al.*, 1997). Phosphorylation of SHP-2 at either tyrosine 304 (pYINA) or 542 (pYTNI) creates potential Grb2 SH2 recognition motifs (pYXNX) (Songyang *et al.*, 1994). In response to PDGF, SHP-2 becomes phosphorylated on tyrosine 542 and associates with Grb2 (Bennett *et al.*, 1994; Li *et al.*, 1994). Thus SHP-2 may play a positive role in signal transduction by functioning as an adaptor molecule, linking Grb2 to the activated PDGFR, providing a mechanism for activation of the Ras/MAP kinase pathway (see section I.H.1d). SHP-2 has also been reported to participate in similar interactions in response to Epo (Tauchi *et al.*, 1995) and SLF (Tauchi *et al.*, 1994) where tyrosine phosphorylated SHP-2 has been shown to directly associate with both Grb2 and the activated receptors. Grb2 has also been shown to associate with tyrosine phosphorylated SHP-2 in response to IL-3, therefore suggesting a

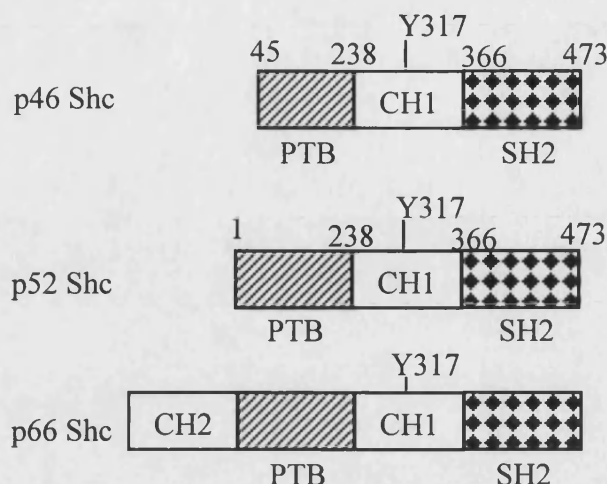
similar adaptor function for SHP-2 in this system (Welham *et al.*, 1994b). In addition, SHP-2 has also been linked with the PI3-K pathway. SHP-2 and PI3-K can be co-precipitated after IL-3 stimulation (Welham *et al.*, 1994b). This association has been shown to be mediated by a 100 kDa protein which can directly interact with both the SH2 domains of the p85 subunit of PI3-K and SHP-2 in response to IL-3 (Craddock and Welham, 1997) and M-CSF (Carlberg *et al.*, 1997). This p100 protein has recently been cloned and is now referred to as Gab2 (Gu *et al.*, 1998). Thus, SHP-2 may function as an adaptor, localising the p100:PI3-K complex to the plasma membrane.

### I.G.2 Shc

In the search for new SH2 domain-containing genes to help define the mechanisms through which tyrosine kinases regulate normal cell growth and induce transformation, a DNA probe representing the *c-fes* SH2 domain was used to screen a human cDNA library prepared from Burkitt lymphoma mRNA and Shc was isolated (Pelicci *et al.*, 1992). This SH2 domain-containing molecule was found to also contain an adjacent glycine/proline-rich motif similar to that of  $\alpha$ 1-collagen, hence the name Shc (src homolgy and collagen-like). The Shc gene encodes three different Shc isoforms of 46, 52 and 66 kDa which contain an amino-terminal PTB domain, a central collagen homolgy domain (CH1), and a carboxy-terminal SH2 domain (See Fig. I.7). The two main forms, p46<sup>Shc</sup> and p52<sup>Shc</sup>, are ubiquitously expressed and result from alternative translational start sites from the same message, resulting in proteins which differ at their amino-terminus which contains a PTB domain. The p46<sup>Shc</sup> isoform lacks the first 45 amino acids of the PTB domain. The p66<sup>Shc</sup> isoform is expressed from a distinct transcript, derived through alternative splicing, and contains an additional region of collagen homology in its unique amino-terminal region, termed the CH2 domain (Migliaccio *et al.*, 1997). p66<sup>Shc</sup> is less widely expressed than the other isoforms, found primarily in epithelial cells and not detected in most human haemopoietic cell lines (Pelicci *et al.*, 1992).



FIGURE I.7



### The different isoforms of Shc.

p46<sup>Shc</sup> and p55<sup>Shc</sup> are expressed from alternative translational start sites from the same message, resulting in proteins which differ at their amino-terminal PTB domain. The p66<sup>Shc</sup> isoform is expressed from a distinct transcript.

Tyrosine phosphorylation of Shc proteins has been shown to occur following activation of receptor tyrosine kinases by growth factors including EGF (Pelicci *et al.*, 1992), PDGF (Yokote *et al.*, 1994), insulin (Pronk *et al.*, 1993; Skolnik *et al.*, 1993) and nerve growth factor (NGF) (Borrello *et al.*, 1994; Stephens *et al.*, 1994). In addition, Shc has been shown to be tyrosine phosphorylated by non-receptor tyrosine kinases in response to cytokines, including: IL-3, IL-5, GM-CSF, SLF, and Epo (Cutler *et al.*, 1993; Damen *et al.*, 1993; Welham *et al.*, 1994a; Matsuguchi *et al.*, 1994; Dorsch *et al.*, 1994; and Lanfrancone *et al.*, 1995). Shc is also phosphorylated in cells transformed by *v-src*, *v-fps* (McGlade *et al.*, 1992) and polyoma virus middle T (mT)-antigen (Dilworth *et al.*, 1994), suggesting that Shc may participate in the transforming activity of oncogenic tyrosine kinases. Phosphorylation of Shc occurs predominantly at tyrosine 317 in the motif pYVNV within the collagen-like domain, providing a high affinity binding site for the SH2 domain of the adaptor protein Grb2 (Salcini *et al.*, 1994).

Shc proteins have been shown to associate with activated receptors. This association is mediated by the Shc SH2 and/or PTB domains. The SH2 domain of Shc has

been shown to recognise phosphorylated tyrosine residues in the context pY[I/E/Y]X[I/L/M], where X is any amino acid (Songyang *et al.*, 1994), whereas the PTB domain binds phosphotyrosine residues in the motif NXXpY (Lamiet *et al.*, 1996). The PTB domain has been shown to direct the association of Shc to activated EGF, HER2/neu and TrkA receptors (Blaikie *et al.*, 1994; Dikic *et al.*, 1995), the insulin receptor (Gustafson *et al.*, 1995) and the IL-3R  $\beta$ c subunit (Pratt *et al.*, 1996). Alternatively, Shc has also been shown to bind to the activated EGFR (Pelicci *et al.*, 1992) and PDGFR (Roche *et al.*, 1996; Yokote *et al.*, 1994) through its SH2 domain. Shc has also been shown to associate via its SH2 domain with the GM-CSFR  $\beta$ c subunit (Lanfrancone *et al.*, 1995), and the EpoR (Damen *et al.*, 1993).

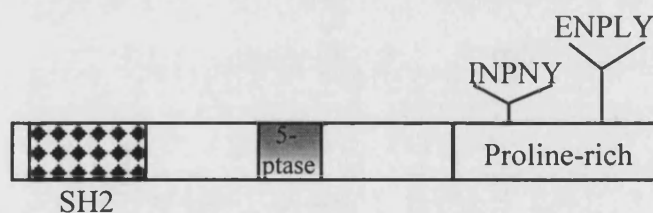
The binding of Shc to the EGFR via its PTB (Blaikie *et al.*, 1994) and SH2 (Pelicci *et al.*, 1992) domains has been shown to result in significant relocation of Shc-Grb2-Sos complexes to the plasma membrane following, EGF treatment (Ruff-Jamison *et al.*, 1993), and hence to the vicinity of Ras. Therefore, it has been suggested that Shc is important in mediating protein-protein interactions leading to the activation of Ras (see section I.H.1d). Additional studies also support this role of Shc. In 3T3 fibroblasts, overexpression of Shc has lead to transformation of mouse fibroblasts and formation of tumours in nude mice (Pelicci *et al.*, 1992), a function found to depend on the presence of the Grb2 SH2 binding site at tyrosine 317 of Shc (Salcini *et al.*, 1994). Mutation of tyrosine 317 to phenylalanine not only resulted in Shc losing the capacity to be highly tyrosine phosphorylated and to bind Grb2, but also to induce transformation (Salcini *et al.*, 1994). Neuronal differentiation of PC12 cells induced by overexpression of Shc was prevented by the co-expression of a dominant inhibitory Ras (N17Ras) mutant, again indicating that Shc is involved in Ras activation (Rozakis-Adcock *et al.*, 1992).

### I.G.3 SHIP

Shc has also been shown to associate, via its PTB domain, with a tyrosine phosphorylated 145 kDa protein from fibroblasts stimulated with PDGF (Kavanaugh and Williams, 1994). This 145 kDa protein becomes tyrosine phosphorylated and associates with Shc in response to multiple cytokines in haemopoietic cells including IL-3, SLF, or Epo stimulation of erythroid cells and megakaryocytes (Cutler *et al.*, 1993; Damen *et al.*, 1993; Lioubin *et al.*, 1994; Liu *et al.*, 1994). The cDNA for this 145 kDa protein was

obtained by using a yeast two-hybrid system based on the affinity of the PTB domain of Shc (Lioubin *et al.*, 1996) and was concurrently cloned by Damen *et al.* (1996). Based on its predicted amino acid sequence, p145 contains an amino-terminal SH2 domain, two PTB binding consensus sequences (INPNY and ENPLY), several proline-rich SH3 binding regions and two motifs highly conserved among inositol polyphosphate 5-phosphatases (see Fig. I.8), hence, this protein was termed SHIP for SH2-containing inositol phosphatase (Damen *et al.*, 1996; Lioubin *et al.*, 1996). SHIP was shown to selectively hydrolyse the 5'-phosphate from inositol 1,3,4,5-tetrakisphosphate and phosphoinositide 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) (Damen *et al.*, 1996; Lioubin *et al.*, 1996).

**FIGURE I.8**



**Schematic representation of the various domains of SHIP.**

SHIP contains an amino-terminal SH2 domain, two PTB binding consensus sequences (INPNY and ENPLY), several proline-rich SH3 binding regions and two motifs highly conserved among inositol polyphosphate 5-phosphatases (indicated by 5-ptase).

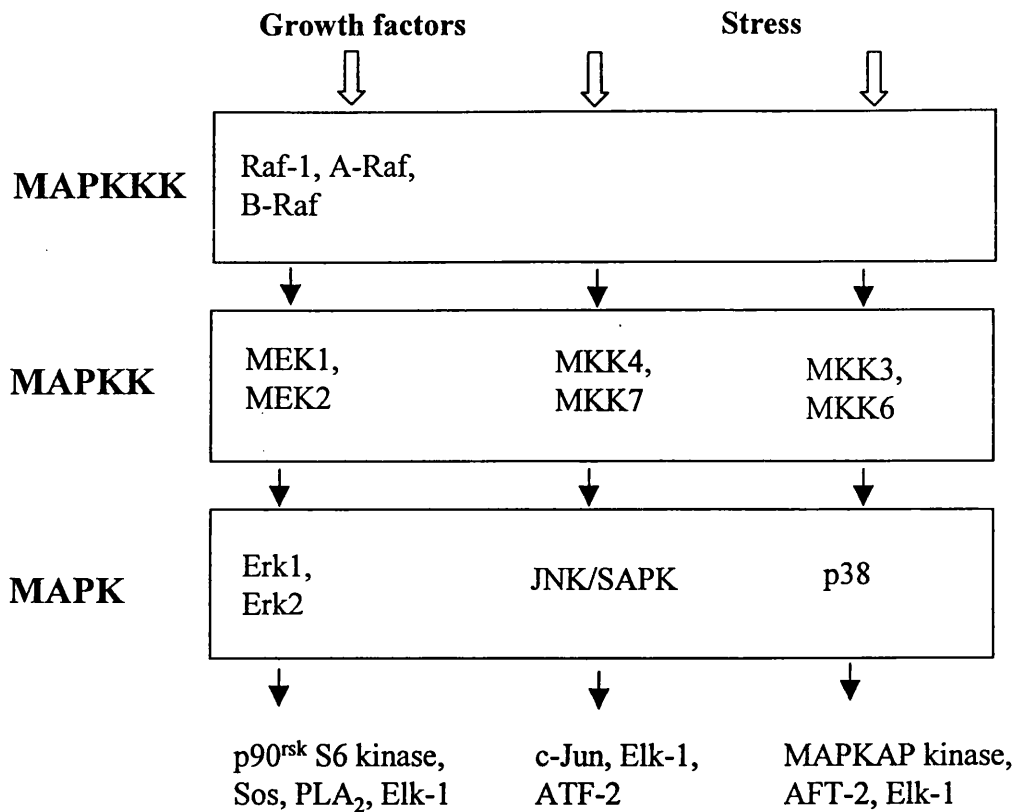
## **I.H Signalling pathways activated by IL-3**

The cytokine IL-3 is an important regulator of haemopoiesis through the modulation of proliferation, differentiation and survival of various haemopoietic lineages and their precursors. These effects are mediated through activation of various signalling pathways. The binding of IL-3 to its receptor rapidly induces activation of multiple signalling pathways, most notably, the Ras/MAP kinase, PI3-K and Jak/STAT pathways.

### **I.H.1 The Ras/MAP kinase pathway**

The Ras/Raf/MEK/ERK pathway is the classical example of what are generically termed mitogen-activated protein kinase (MAP kinase) pathways. MAP kinase pathways have as their “core” a three-component protein kinase cascade consisting of a serine/threonine MAP kinase kinase kinase (MAPKKK), which phosphorylates and activates a dual-specificity MAP kinase kinase (MAPKK). MAPKK subsequently phosphorylates, at both tyrosine and threonine residues, and activates the serine/threonine protein kinase, MAP kinase. These pathways serve to link signals from the cell surface to cytoplasmic and nuclear events.

In addition to the classical MAP kinase cascade leading to activation of the MAP kinases erk1 and erk2 (discussed below), there exist cascades leading to activation of two additional members of the MAP kinase family: c-Jun amino-terminal kinase (JNK) and p38 MAP kinase (see Fig. I.9). Briefly, JNK, also known as stress-activated protein kinase (SAPK), is activated in response to environmental stress, including changes in osmolarity, UV radiation, heat shock and DNA damage, as well as growth factors and pro-inflammatory cytokines (reviewed by Ip and Davis, 1998). JNK activation is mediated by dual phosphorylation on threonine and tyrosine residues, within a Thr-Pro-Tyr motif, by two MAPKKs, MKK4 and MKK7. Activated JNK, in turn, phosphorylates and activates transcription factors including c-Jun, ATF-2 and Elk-1 (Whitmarsh and Davis, 1996). Both c-Jun and ATF-2 form functional heterodimers or homodimers at AP-1 consensus binding sites, thereby facilitating the activation of gene transcription (Kallunki *et al.*, 1996). Like JNK, p38 MAP kinase is activated by cellular stress as well as the pro-inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and IL-1. Activation is mediated by dual phosphorylation at the Thr-Gly-Tyr motif by the MAPKKs, MKK3 and MKK6.

**FIGURE I.9**

### Mitogen-activated protein kinase cascades.

The MAP kinase cascades leading to activation of the MAP kinases erk, JNK/SAPK, and p38 are illustrated schematically. The MAPKKs MEK1 and MEK2 activate the erk subgroup of MAP kinases, typically in response to growth factors. The MAPKKs MKK4, MKK7, MKK3, and MKK6 activate the JNK/SAPK and p38 MAP kinases, as indicated, in response to stress signals. Once activated, the MAP kinases can activate transcription factors (Elk-1, c-Jun, ATF-2), other kinases (p90<sup>rsk</sup> S6 kinase, MAPKAP kinase), upstream regulators (Sos), and other regulatory enzymes (PLA<sub>2</sub>). These downstream targets then control cellular responses including growth and differentiation.

Activated p38 subsequently phosphorylates and activates transcription factors, including ATF-2 and Elk-1. In addition, p38 activates MAPKAP kinase-2 which, in turn phosphorylates the small heat shock proteins Hsp25 and Hsp27 (Rouse *et al.*, 1994).

The classical Ras/Raf/MEK/Erk pathway is activated in response to cytokine stimulation. As will be discussed below, cytokine binding to a cell surface receptor leads to activation of Ras, facilitated by promotion of guanine nucleotide exchange on Ras. Ras activation subsequently leads to activation of the MAPKKK, Raf, followed by activation of the MAPKKs, MEK1 and MEK2, and finally to activation of the MAP kinases Erk1 and Erk2.

### I.H.1a Ras

The mammalian genome consists of at least three *ras* proto-oncogenes, designated c-H-*ras*, c-K-*ras*, and c-N-*ras*. All three cellular *ras* genes have pronounced oncogenic potential. The *ras* genes were first identified as the transforming agents of the Harvey and Kirsten rat sarcoma viruses (Harvey, 1964; Kirsten and Mayer 1967). These viral *ras* oncogenes (v-H-*ras* and v-K-*ras*) have the ability to transform fibroblasts in cell culture and induce sarcomas and erythroleukemias in susceptible mice. c-N-*ras* was identified as a dominant transforming gene of a human neuroblastoma cell line (Shimizu *et al.*, 1983). In fact, nearly one third of human tumours have been shown to express activated versions of the *ras* family of genes, suggesting a role for Ras in regulating cell proliferation. The *ras* gene products are collectively referred to as p21<sup>ras</sup> (Barbacid, 1987; Lowry and Willumsen 1993). The Ras proteins are 21 kDa plasma-membrane associated proteins which bind guanine nucleotides and have intrinsic GTPase activity. Association of Ras with the inner side of the plasma membrane requires a post-translational modification that involves acylation of Cys<sup>186</sup> by palmitic acid (Buss and Sefton, 1986).

Ras proteins act as regulatory switches whose activity is controlled by cycling between an active guanosine triphosphate (GTP)-bound and an inactive guanosine diphosphate (GDP)-bound state (see Fig. I.10) (Polakis and McCormick, 1993). In this Ras GTPase cycle, Ras-GDP is converted to Ras-GTP by the exchange of bound GDP with GTP, while hydrolysis of bound GTP to GDP regenerates Ras-GDP from Ras-GTP. Thus, Ras proteins are regulated in two ways. Guanine nucleotide exchange factors (GNEF; or guanine nucleotide release factors), such as Sos and Ras-GRF, accelerate the exchange of

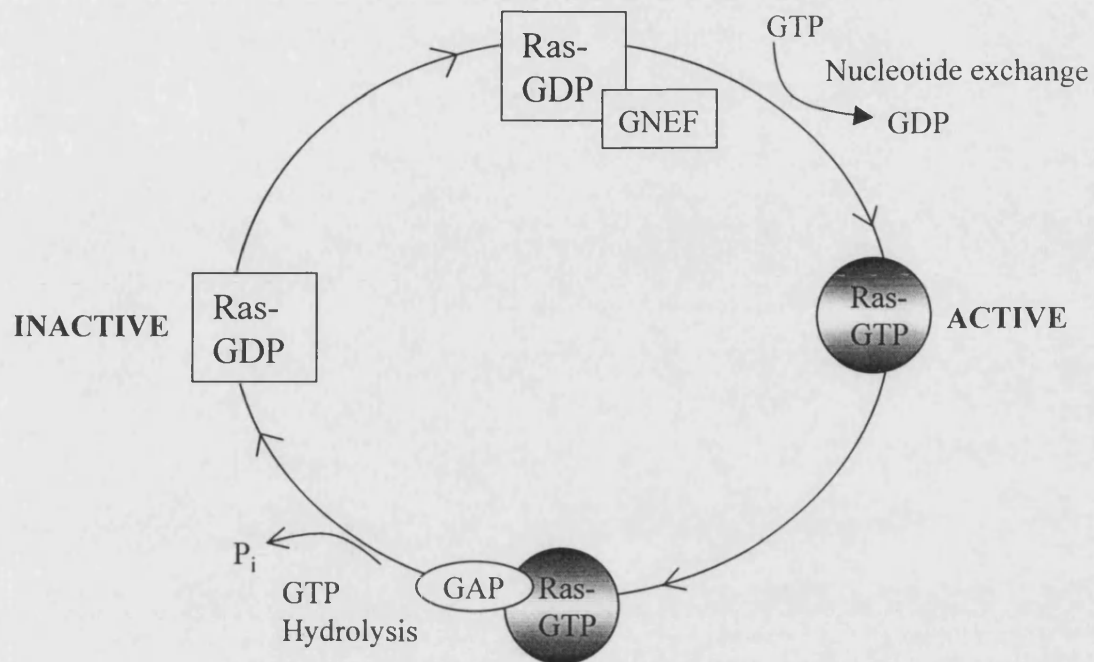
GDP for GTP and hence activate Ras (reviewed by Feig, 1993). While Ras GTPase activating proteins, such as p120GAP (Trahey and McCormick, 1987) and neurofibromin (NF-1) (Xu *et al.*, 1990), increase the intrinsic GTPase activity of Ras, thereby negatively regulating Ras function.

Ras functions as a crucial mediator of many biological responses, including proliferation and differentiation, stimulated by both receptor and non-receptor tyrosine kinases (reviewed by Satoh *et al.*, 1992). Ras has been shown, by both direct and indirect approaches, to be involved in events downstream of tyrosine kinases. Microinjection of the neutralising anti-Ras monoclonal antibody Y13-259 into fibroblast cells inhibited DNA synthesis induced by serum, PDGF and EGF (Mulcahy *et al.*, 1985) and inhibited transformation by tyrosine kinase encoding oncogenes (Smith *et al.*, 1986). Expression of a dominant inhibitory mutation in the c-H-*ras* gene, which changes Ser-17 to Asn-17 in the gene product (N17-Ras), inhibits proliferation of NIH 3T3 cells (Feig and Cooper, 1988). Dominant negative mutants of Ras also blocked DNA synthesis or gene expression induced by EGF and insulin (Cai *et al.*, 1990; Medema *et al.*, 1991). Several studies have implicated Ras in signal transduction pathways stimulated by both non-receptor and receptor tyrosine kinases in lymphoid and myeloid cell lines as measured by an increase in the amount of Ras having bound GTP compared to GDP. An accumulation of Ras-GTP was observed after stimulation of various B cell, T cell and mast cell lines with cytokines including IL-3, SLF, GM-CSF (Duronio *et al.*, 1992b; Satoh *et al.*, 1991), EGF (Satoh *et al.*, 1990a), PDGF (Satoh *et al.*, 1990b), insulin (Burgering *et al.*, 1991) and T cell receptor cross linking (Downward *et al.*, 1990).

### **I.H.1b Son of Sevenless (Sos)**

Activation of Ras is accomplished by GNEFs which accelerate the exchange of GDP for GTP. The first guanine nucleotide exchange factor for Ras, CDC25, was identified in yeast *Saccharomyces cerevisiae* and was shown to be essential for activation of Ras proteins (Broek *et al.*, 1987). In *Drosophila*, the protein encoded by the *son of sevenless* gene, Sos, contained a domain that showed sequence similarity to the catalytic domain of CDC25 (Simon *et al.*, 1991; Bonfini *et al.*, 1992). Sos was shown to function downstream from both the sevenless receptor tyrosine kinase and the *Drosophila* EGF receptor homologue (DER) (Simon *et al.*, 1991; Rogge *et al.*, 1991). Two related murine

FIGURE I.10



### Cycling of p21<sup>ras</sup> between GTP and GDP bound states.

In its inactive state, Ras is bound to GDP. Guanine nucleotide exchange factors (GNEF) accelerate the exchange of GDP for GTP, activating Ras to its GTP-bound state. GTPase activating proteins (GAPs) increase the intrinsic GTPase activity of Ras, converting the active GTP-bound Ras, back to the inactive GDP-bound state.



genes, designated mouse Son of sevenless 1 and 2 (mSos1 and mSos2), with extensive homology to *Sos* were later identified (Bowtell *et al.*, 1992). Both mSos1 and mSos2 are widely expressed during development and in adult tissues (Bowtell *et al.*, 1992). The human cDNA, encoding a widely expressed human protein hSos1, was further isolated and was also closely related to *Sos* (Chardin *et al.*, 1993).

In fibroblasts, the mSos1 protein was shown to act as a specific guanine nucleotide exchange factor for Ras (Buday and Downward, 1993a). mSos1 can increase GTP loading on mammalian Ras (Egan *et al.*, 1993). However, the guanine nucleotide exchange activity of mSos1 was not affected by EGF treatment (Buday and Downward, 1993a; Gale *et al.*, 1993), even though growth factor treatment had been found to increase the rate of nucleotide exchange on Ras (Buday and Downward 1993b). The Grb2 adaptor protein (see section I.H.1c) was also found to associate with mSos1 and the EGFR following EGF stimulation (Buday and Downward, 1993a). Thus, it was suggested that stimulation of the receptor serves to translocate a Grb2-Sos complex to the receptor and into the proximity of its target, membrane-bound Ras. In support of this mechanism is the observation that targeting of *Sos1* to the plasma membrane is sufficient for the activation of the Ras signalling pathway (Quilliam *et al.*, 1994; Aronheim *et al.*, 1994).

Growth factor stimulation also induces phosphorylation of *Sos* on serine and threonine residues (Cherniak *et al.*, 1994; Rozakis-Adcock *et al.*, 1993). Phosphorylation occurs predominantly at the carboxy-terminus and is mediated by the MAP kinases erk1 and erk2. This phosphorylation appears to result in its dissociation from Grb2 and may constitute a negative feedback mechanism to control Ras activity (Corbalan-Garcia *et al.*, 1996).

### **I.H.1c Growth factor receptor-bound protein 2 (Grb2)**

Grb2 was cloned whilst screening for proteins that bound tyrosine phosphorylated EGF receptors. The tyrosine-autophosphorylated carboxy-terminus tail of the EGFR was used to probe a bacterial cDNA expression library for novel EGFR-binding proteins (Lowenstein *et al.*, 1992). Grb2 is a 23 kDa, widely expressed protein whose entire sequence is composed of a single SH2 domain flanked by two SH3 domains (see Fig. I.4). Grb2 is the mammalian homologue of the *Caenorhabditis elegans* protein sem-5 which functions downstream of the Let-23 receptor tyrosine kinase and upstream of the Let-60

Ras protein (Clark *et al.*, 1992). Drk is the *Drosophila* protein that is identical in structure to sem-5 and Grb2, and functions downstream of the sevenless receptor tyrosine kinase (Simon *et al.*, 1993).

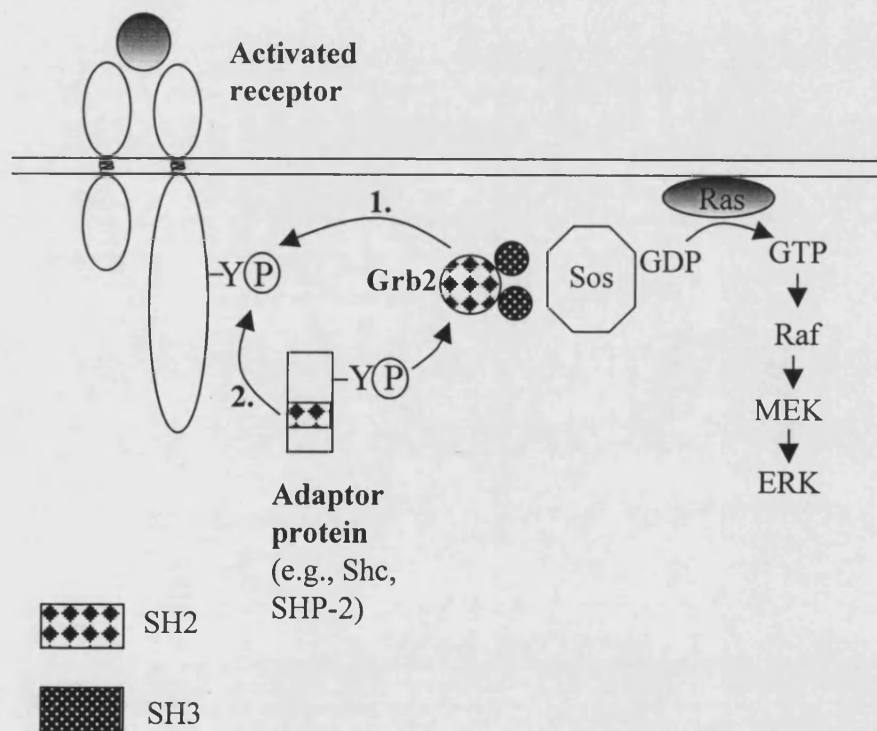
Since Grb2 has no intrinsic catalytic activity, it functions as an adaptor protein, linking other signalling molecules together. In most cells, Grb2 is constitutively associated with Sos1. This association is mediated through binding of the two Grb2 SH3 domains to the proline-rich carboxy-terminal tail of Sos1 which contains the consensus sequence VPVPPPVP (Rozakis-Adcock *et al.*, 1993; Li *et al.*, 1993; Egan *et al.*, 1993). Point mutations in either SH3 domain of sem-5 resulted in loss-of-function alleles (Clark *et al.*, 1992). Similar SH3 mutations in human Grb2 SH3 domains also impaired its function, resulting in loss of Sos1 binding (Lowenstein *et al.*, 1992), indicating that high-affinity binding to Sos1 requires both Grb2 SH3 domains.

#### **I.H.1d Protein-protein interactions controlling Ras activation**

Regulation of Ras activation is largely mediated through translocation of the Grb2-Sos complex to the plasma membrane and hence the vicinity of Ras (see Fig. I.11). A number of important protein-protein interactions have been characterised which are responsible for this relocalisation. Grb2 can directly or indirectly associate with activated growth factor receptors. The SH2 domain of Grb2 can directly associate with amino acids surrounding tyrosine 1068 of the EGF receptor (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1993; Buday and Downward, 1993a). However, for many receptors Grb2 does not bind directly but instead associates with an adaptor protein which is directly bound to the activated receptor, thus providing a link between the Grb2-Sos complex and the receptor. One such adaptor protein is Shc. Grb2, via its SH2 domain, has been shown to associate with residues surrounding tyrosine 317 of Shc (Salcini *et al.*, 1994). Shc itself can simultaneously associate with activated receptors via its SH2 and/or PTB domain (discussed in section I.G.2), thereby linking the Grb2-Sos complex to the activated receptor. In the case of the EGF receptor, as well as interacting directly, Grb2 can also indirectly associate with the EGF receptor via Shc. In response to EGF, significant relocation of Shc-Grb2-Sos complexes to the plasma membrane have been observed, mediated by such interactions (Ruff-Jamison *et al.*, 1993). The PTPase SHP-2 also acts as an adaptor molecule, facilitating the localisation of Grb2-Sos complexes to activated

receptors. The Grb2 SH2 domain has been shown to associate with tyrosine phosphorylated SHP-2 and SHP-2, itself, can also directly associate, via its SH2 domain, with activated receptors (see section I.G.1b). Thus, the direct binding of adaptor proteins such as Shc and SHP-2 to activated receptors, and the simultaneous association with Grb2, via its SH2 domain, with these adaptor proteins, provides a means of localising Grb2-Sos complexes to the plasma membrane and hence to the vicinity of Ras, leading to its activation.

**FIGURE I.11**



### **Association of Grb2-Sos complexes with activated receptors.**

Regulation of Ras activation is mediated through translocation of the Grb2-Sos complex to the membrane. This is accomplished through either (1) direct association of Grb2 with activated receptors, or (2) association with an adaptor protein, such as SHP-2 or Shc, which directly associates with the receptor.

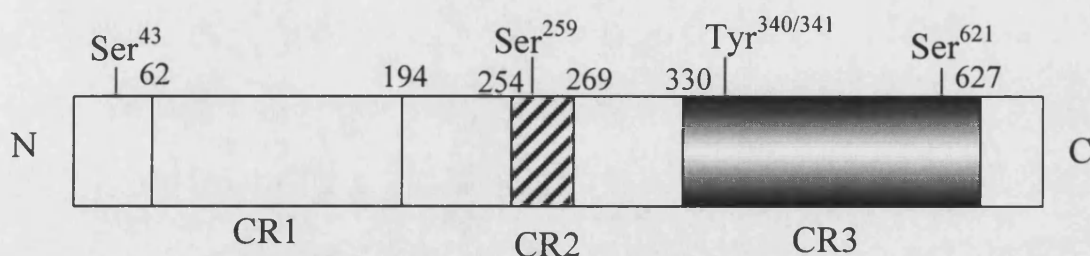
### I.H.1e Raf-1

Raf-1 is a ubiquitously expressed 72-76 kDa cytoplasmic protein with intrinsic serine/threonine kinase activity. The *raf-1* gene was first identified as the normal cellular counterpart of *v-raf*, the transforming gene of murine sarcoma virus 3611 (Rapp *et al.*, 1983). *v-Raf* is expressed as a myristylated Gag-Raf fusion protein consisting of the amino-terminal 384 amino acids of Gag and the carboxy-terminal 323 residues of Raf (Rapp *et al.*, 1983). Two closely related members of the Raf family are also expressed in mammalian cells: A-Raf (Beck *et al.*, 1987) and B-Raf (Eychene *et al.*, 1992). In mammalian cells, Raf-1 has been shown to function downstream of Ras, as expression of a dominant inhibitory mutant or anti-sense mRNA of Raf-1 inhibits *v-ras*-induced transformation of fibroblasts (Kolch *et al.*, 1991). In addition, *v-raf* transformed cells are unaffected by injection of an inactivating anti-Ras antibody (Smith *et al.*, 1986).

The Raf-1 protein is composed of three conserved domains: CR1, CR2 and CR3 (Fig. I.12). The CR1 region, located in the amino-terminal portion of the molecule (amino acids 62 to 194), is rich in cysteine residues and contains a putative zinc binding region (Berg *et al.*, 1986). CR2, also located in the amino-terminal portion of the Raf protein (amino acids 254 to 269), is a region rich in serine and threonine residues. CR3, in the carboxy-terminal portion of the protein (amino acids 330 to 627), contains the protein kinase domain. Experimental evidence suggests that the amino-terminal domain, containing CR1 and CR2, functions to regulate the catalytic activity of Raf-1 as deletion or mutation of this region activates Raf-1 transforming activity (Stanton *et al.*, 1989; Heidecker *et al.*, 1990). In addition, the integrity of the CR1 domain is crucial for normal regulation of Raf-1 function, and for the ability of the amino-terminal domain (amino acids 1-257) to act as a dominant inhibitor of Ras (Bruder *et al.*, 1992). Using the yeast two hybrid system and *in vitro* binding assays, it has been demonstrated that the amino-terminal CR1 domain of Raf-1 can directly interact with the Ras effector domain (amino acids 32-40 of Ras) and is dependent on Ras being in its GTP-bound state (Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). Interestingly, Raf-1 and the two Ras GTPase-activating proteins, p120GAP and NF-1, probably compete for Ras-GTP in the cell since they all bind to the same effector region. However, this direct association with Ras does not lead to Raf-1 activation. Instead, it appears that recruitment to the plasma membrane is necessary to activate Raf-1, suggesting that the role of Ras is to recruit Raf-1 to the plasma

membrane. Experiments suggest that Ras-mediated translocation of Raf-1 to the cytoplasmic membrane is a crucial step in Raf-1 activation (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). However, localisation to the membrane is necessary but not sufficient for the stable activation of Raf-1.

**FIGURE I.12**



### **Schematic representation of Raf-1.**

The three regions that are highly conserved among Raf proteins, namely conserved region (CR) 1, CR2, and CR3 are shown. CR1 is rich in cysteine residues; CR2 is rich in serine/threonine residues; and CR3 is the kinase domain. Ser<sup>43</sup>, Ser<sup>259</sup>, and Ser<sup>621</sup> are *in vivo* sites of serine phosphorylation and Ser<sup>259</sup> and Ser<sup>621</sup> each constitute part of the consensus motif for 14-3-3 binding. Phosphorylation of Tyr<sup>340/341</sup> enhances the catalytic activity of Raf.

The 14-3-3 proteins have been implicated in the regulation of Raf. 14-3-3 proteins are 30 kDa phosphoserine-binding proteins which recognise the sequence motifs RXXSpSXP (where X is any amino acid and pS is the phosphorylated serine) (Muslin *et al.*, 1996). 14-3-3 protein were isolated in a yeast two-hybrid screen for Raf-1 kinase domain binding proteins (Li *et al.*, 1995). How 14-3-3 protein regulate Raf-1 activity is poorly understood and there are differing reports as to the effect these proteins have on Raf-1. The interaction between 14-3-3 and Raf-1 appears to be functional as

overexpression of 14-3-3 in fibroblast cells resulted in Raf-1 activation (Li *et al.*, 1995). However, 14-3-3 proteins have been shown to be bound constitutively to Raf-1 in unstimulated cells (Li *et al.*, 1995) and were found to associate in part via a sequence surrounding phosphorylated Ser-621, a Raf-1 site which is constitutively phosphorylated (Muslin *et al.*, 1996). There is also evidence that Ser-259, whose phosphorylation is only induced after growth factor stimulation, is also a 14-3-3 binding site (Michaud *et al.*, 1995). Thus, binding of 14-3-3 does not directly activate Raf-1 as Raf-14-3-3 complexes are present in unstimulated cells (Li *et al.*, 1995). However, mutation of serine 621 in Raf-1 inhibits the association of 14-3-3 (Michaud *et al.*, 1995) and renders Raf-1 inactive. Therefore, 14-3-3 binding to serine 621 may be required for Raf-1 kinase activity. Recently, a model has been proposed for the role of 14-3-3 in the regulation of Raf activity (Tzivion *et al.*, 1998). Raf is maintained in an inactive state by the binding of a 14-3-3 dimer to a single Raf polypeptide at two sites, phosphorylated Ser 259 and Ser 621. Ras-GTP subsequently displaces 14-3-3 from phosphorylated Ser 259. Finally, half of the 14-3-3 dimer displaced from phosphorylated Ser 259 by Ras-GTP rebinds to a putative new phosphoserine site, thereby stabilising an active conformation that no longer requires Ras-GTP. However, the exact mechanism by which 14-3-3 activates Raf is not fully understood and is still under investigation.

Another protein which may be involved in Raf-1 activation is the kinase suppressor of Ras (Ksr). Ras activation has been shown to induce translocation of Ksr from the cytoplasm to the membrane where it activates Raf-1 activity (Therrien *et al.*, 1995). Interestingly, Ksr enhancement of Raf-1 activity is independent of Ksr kinase activity. Yeast two-hybrid analysis using mouse Ksr as bait showed interactions of Ksr with both MEK and ERK (Yu *et al.*, 1997), thus Ksr might also have a scaffold function, bringing together specific components of the MAP kinase cascade, in regulating the Raf-MEK-ERK pathway.

The observation that Raf-1 becomes hyperphosphorylated in response to many signalling events (Morrison *et al.*, 1993) has suggested that phosphorylation plays a role in regulating Raf-1 activity. Mechanisms by which phosphorylation could regulate Raf-1 function include directly altering Raf-1 intrinsic kinase activity and mediating critical protein-protein interaction, such as with 14-3-3. In mammalian cells, the major sites for serine phosphorylation are Ser<sup>43</sup>, Ser<sup>259</sup>, and Ser<sup>621</sup> (Morrison *et al.*, 1993). Both Ser<sup>43</sup> and

Ser<sup>621</sup> are phosphorylated in unstimulated and PDGF stimulated cells and mutation of Ser<sup>621</sup>, which is located in the CR3 kinase domain, inactivates the biochemical activity of the kinase (Morrison *et al.*, 1993). Ser<sup>259</sup>, which is located in the serine/threonine rich CR2 region, was phosphorylated only after stimulation with PDGF and mutation resulted in an activated Raf-1 protein, suggesting that this residue functions in a negative regulatory way (Morrison *et al.*, 1993). Phosphorylation of tyrosine residues 340 and 341 has been shown to enhance the catalytic activity of Raf-1 (Fabian *et al.*, 1993; Marais *et al.*, 1995).

Downregulation of Raf activity may be a result of a feedback phosphorylation by a downstream component of the pathway, the MAP kinases erk1 and erk2. Erks have been shown to phosphorylate Raf-1 and it has been suggested that this may play a negative regulatory role (Anderson *et al.*, 1991; Lee *et al.*, 1992; Williams *et al.*, 1993; Samuels *et al.*, 1993).

### I.H.1f MEK

Raf-1 has been shown to phosphorylate and activate MEK (MAP kinase/Erk-activating kinase). At least two isoforms of MEK, MEK1 and MEK2, exist in mammalian cells, and these catalyse the dual phosphorylation on threonine and tyrosine residues of the MAP kinases erk1 and erk2 (see section I.H.1g) (Crews *et al.*, 1992; Zheng and Guan, 1993; Wu *et al.*, 1993). Phosphorylation of MEK by Raf occurs on serine residues at positions 217 and 221 which are conserved between MEK1 and MEK2 (Alessi *et al.*, 1994). Activation of MEK appears to require phosphorylation of either serine residue. However, inactivation of MEK requires dephosphorylation of both serine 217 and 221 residues (Alessi *et al.*, 1994). These serines lie between protein kinase subdomains VII and VIII, the same region where the activating phosphorylations for a number of other protein kinases are located (Hanks *et al.*, 1988). The activation of MEK by Raf-1 was demonstrated by three groups who reported that when MEK preparations are inactivated by treatment with a serine/threonine phosphatase, MEK activity can be restored by either oncogenic Raf or cellular Raf proteins from stimulated mammalian cells (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992).

MEK1 and MEK2 contain a proline-rich sequence (PRS) in their carboxy-terminal domains spanning residues 270-307 between kinase subdomains IX and X. Deletion of the PRS from MEK1 impairs its activation by Raf-1 in transfected cells, suggesting that the

insert may be involved in the coupling of MEK1 to components of the MAP kinase cascade (Catling *et al.*, 1995). Recently, using a yeast two-hybrid screen, a protein called MP1 (MEK Partner 1) was identified that bound specifically MEK1 and erk1 and facilitated their activation (Schaeffer *et al.*, 1998). The PRS sequence within MEK1 was shown to be necessary for its association with MP1. It has been suggested that MP1 functions to increase the efficiency of Raf activation, as addition of MP1 to an *in vitro* assay with purified recombinant B-Raf and MEK1 enhanced MEK phosphorylation (Schaeffer *et al.*, 1998).

MEK1 and MEK2 exhibit considerable similarity within the PRS. However, there are two potential phosphorylation sites unique to MEK1 at threonine 286 and 292 (Wu *et al.*, 1993a,b). Threonine 292 is likely to play an important role in directing protein-protein interactions as a mutant MEK1 protein (T292A) was unable to bind Ras suggesting that phosphorylation on threonine 292 controls the binding of MEK1 to the Ras signaling complex (Jelinek *et al.*, 1994) and is likely to be involved in binding MEK1 to MP1. Interestingly, the MAP kinase, erk1, has been shown to be able to phosphorylate MEK1 on threonine 292 as well as threonine 386 which is conserved in MEK2. (Brunet *et al.*, 1994; Saito *et al.*, 1994). Phosphorylation on threonine 386 has been suggested to serve as a negative feedback control, reducing MEK1 activity (Brunet *et al.*, 1994).

### **I.H.1g Extracellular-signal-regulated kinases (Erks)**

Activation of a family of intracellular serine/threonine kinases referred to as the mitogen activated protein kinases (MAPKs) or extracellular-signal-regulated kinases (erks) has long been associated with regulation of proliferation and differentiation. Among the MAP kinases found in mammalian cells are two highly homologous MAP kinases which are expressed ubiquitously: p42<sup>mapk</sup>, or erk2 and p44<sup>mapk</sup>, or erk1 (Boulton *et al.*, 1991). MEK1 and MEK2 activate erk1 and erk2 by phosphorylation on both threonine 183 and tyrosine 185 within a conserved MAP kinase sequence motif Thr-Glu-Tyr adjacent to subdomain VIII of the kinase domain (Payne *et al.*, 1991; Hanks *et al.*, 1988). Growth factor stimulation of cells results in the translocation of both erk1 and erk2 to the nucleus approximately 15 minutes after stimulation and growth factor removal rapidly reverses this process of nuclearisation and abolishes erk activation (Lenormand *et al.*, 1993). Thus, erks



provide a physical link in the signal transduction pathway from the cytoplasm to the nucleus.

MAP kinases are proline-directed protein kinases that phosphorylate the consensus sequence Pro-X-Ser/Thr-Pro (Gonzalez *et al.*, 1991). Erk1 and erk2 have many potential substrates including further downstream kinases as well as transcription factors. Substrates phosphorylated and subsequently activated by erks include the cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) (Lin *et al.*, 1993) and the ribosomal S6 kinases rsk1 and rsk2 (p90<sup>rsk</sup>) (Sturgill *et al.*, 1988) (see Fig. I.9). cPLA<sub>2</sub> represents an important target of the MAP kinase signal transduction pathway. Phosphorylation at Ser<sup>505</sup> causes an increase in the enzymatic activity of cPLA<sub>2</sub>, resulting in increased arachidonic acid release and formation of lysophospholipids from membrane phospholipids (Lin *et al.*, 1993). Activation of p90<sup>rsk</sup> phosphorylates the 160 kDa glycogen binding (G) subunit of protein phosphatase I (PP-I). This phosphorylation increases the binding of the catalytic subunit of PP-I to the glycogen bound G subunit and results in increased glycogen synthase phosphatase activity (Dent *et al.*, 1990). This results in an increase in glycogen synthase activity, leading to glycogen synthesis.

Transcription factors are a key target of erk phosphophorylation. Most notably, the ternary complex factor (TCF), Elk-1, which is involved in *c-fos* induction, is phosphorylated by erks on sites essential for transactivation. Phosphorylation of Elk-1 results in formation of a complex with the serum response factor (SRF), which together bind the serum response element (SRE) on the *c-fos* promoter (Marais *et al.*, 1993). *c-fos* is an immediate early gene involved in cellular growth and differentiation. Thus, erk activation is a key event leading to transcriptional activation of *c-fos*, and regulating cell growth and differentiation.

Additional erk substrates include protein kinases that form the cascade that leads to erk activation: c-Raf-1 (Anderson *et al.*, 1991; Lee *et al.*, 1992) and MEK (Matsuda *et al.*, 1993; Brunet *et al.*, 1994), suggesting that this MAP kinase cascade may be regulated by the erks themselves. In addition, Sos has been shown to be phosphorylated by erk, resulting in its dissociation from Grb2 and possibly constituting a negative feedback mechanism (Corbalan-Garcia *et al.*, 1996). Furthermore, following activation of erk, new transcription can lead to the production of specific phosphatases which could act as a timer to turn off MAP kinase activity. The phosphatase CL100 is regulated at the transcriptional

level by growth factors and stress and specifically inactivates MAP kinase *in vivo* (Keyse *et al.*, 1992; Charles *et al.*, 1992; Alessi *et al.*, 1993). More recently, activation of this MAP kinase cascade was shown to promote the induction of two members of a recently described family of dual specificity phosphatases, MKP-1 and MKP-2, which are capable of dephosphorylating MAP kinase and may attenuate MAP kinase-dependent events in an inhibitory feedback loop (Brondello *et al.*, 1997).

### I.H.2 Phosphoinositide 3-kinase (PI3-K)

Phosphoinositide 3-kinase (PI3-K) activity has been implicated in the regulation of a number of different cellular responses including growth. PI3-K is a lipid kinase capable of phosphorylating phosphoinositides at the 3' position of the inositol ring (Stephens *et al.*, 1993). The primary isoform of PI-3K, class IA, become activated in response to stimulation with growth factors, including IL-3, IL-4, SLF, GM-CSF and IL-5 (Gold *et al.*, 1994), and are capable of phosphorylating PI, PI(4)P, and PI(4,5)P<sub>2</sub>, converting them to PI(3)P, PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> respectively. However, their preferred substrate is thought to be PI(4,5)P<sub>2</sub>. The class IA PI3-K are heterodimeric enzymes consisting of an 85 kDa regulatory subunit (p85 $\alpha$  or p85 $\beta$ ) and a 110 kDa catalytic subunit. Three p110 isoforms, p110 $\alpha$ , p110 $\beta$  and p110 $\delta$  have been described (Vanhaesebroeck *et al.*, 1997a), with p110 $\delta$  expression largely restricted to leukocytes (Vanhaesebroeck *et al.*, 1997b). p110 $\alpha$  and p110 $\beta$  appear to be ubiquitously expressed and have previously been shown to be coupled to IL-3 signalling (Vanhaesebroeck *et al.*, 1997b; Gold *et al.*, 1994). The p85 subunit (see Fig. I.4) contains a number of domains that mediate protein-protein interactions including an SH3 domain, two proline rich regions, two SH2 domains and a region with similarity to the break point cluster gene (BCR homology region). The two SH2 domains are separated by the inter-SH2 (iSH2) region. Part (amino acids 478-513) of the iSH2 domain mediates the interaction of p85 with amino acids 20-108 of the amino-terminus of p110 and this interaction is required for regulation of the enzymatic activity of p110 (Klippel *et al.*, 1994; Holt *et al.*, 1994; Dhand *et al.*, 1994).

Regulation of PI3-K activity not only requires interaction of the two subunits, but also membrane localisation. The two SH2 domains of the p85 subunit bind phosphorylated tyrosine residues specifically within a pYXXM motif (Cohen *et al.*, 1995). The tyrosine residues on receptors or associated signalling molecules phosphorylated in response to

ligand binding form the docking sites for the SH2 domains of the p85 subunit. This adaptor-mediated translocation of PI3-K to the activated receptor is likely to help position the catalytic subunits close to the membrane which contains their lipid substrates.

In addition, activation of PI3-K may also require interaction with Ras in a GTP-dependent manner. GTP-bound Ras has been shown to bind the catalytic p110 subunit of PI3-K, resulting in stimulation of PI3-K activity (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994,1996). Co-expression studies of PI3-K with various Ras mutants indicate that Ras can regulate PI3-K *in vivo* (Rodriguez-Viciana *et al.*, 1994; Marte, *et al.*, 1997). Expression of a dominant negative Ras mutant inhibited the ability of NGF and EGF to elevate PI(3,4,5)P<sub>3</sub> lipid levels in PC12 cells and the co-expression of Ras with p85 and p110 in COS cells also resulted in increased cellular 3'phosphorylated phospholipids, implicating Ras in the regulation of PI3-K (Rodriguez-Viciana *et al.*, 1994). Evidence from experiments using PDGF-receptor mutants also suggests that accumulation of GTP-bound Ras is required for activation of PI3-K by PDGF (Klinghoffer *et al.*, 1996). Taken together, these data indicate that PI3-K may be another class of Ras effector molecules and position Ras as an upstream regulator of PI3-K. The interaction of Ras with PI3-K might result in allosteric activation, or contribute to PI3-K recruitment to the plasma membrane. It has been demonstrated that targeting of p110 to the membrane is sufficient for activation of the p70<sup>S6K</sup> and PKB/Akt, but not MAP kinase, in COS cells (Klippel *et al.*, 1996). However, it should also be noted that data have been reported that position Ras downstream of PI3-K (Hu *et al.*, 1995). Expression of a constitutively activated PI3-K mutant, p110\*, in fibroblasts induced transcription of the *fos* promoter which was blocked by expression of dominant negative Ras (Hu *et al.*, 1995). In addition, expression of p110\* in *Xenopus oocytes* resulted in an elevated level of GTP-bound Ras, providing direct evidence that PI3-K can activate the Ras pathway (Hu *et al.*, 1995).

The precise mechanism of PI3-K signalling is not known but studies indicate that this enzyme is linked to several pathways. PI3-K is involved in the activation of the serine/threonine kinase p70<sup>S6K</sup> which is important for mitogenic signals leading to serum-induced protein synthesis, *c-fos* induction and entry into S phase of the cell cycle (Lane *et al.*, 1993; Weng *et al.*, 1995). Another putative downstream effector of PI3-K is the serine/threonine protein kinase PKB/Akt (Burgering and Coffey, 1995; Franke *et al.*, 1995). Activation of PKB/Akt protects certain cells from apoptosis and the mechanism by which

this occurs is currently under intensive investigation. The best candidate mechanism to date is through the phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family, resulting in its binding to 14-3-3 as an inactive complex (Zha *et al.*, 1996; Del Peso *et al.*, 1997; Datta *et al.*, 1997).

PI3-K may also play a role in activation of the MAP kinases erk1 and erk2. Several reports have demonstrated inhibition of MAP kinase activation by wortmanin, a potent inhibitor of PI3-K (Cross *et al.*, 1994; Ferby *et al.*, 1994; Welsh *et al.*, 1994; Von Willebrand *et al.*, 1996; Grammer and Bleni, 1997; Jascur *et al.*, 1997). Importantly, wortmannin was shown to partially inhibit MEK-dependent activation of MAP kinase in response to various cytokines including PDGF, IL-2 and insulin (Grammer and Blenis, 1997). Activation of erk1 by GM-CSF and IL-3 was also shown to be attenuated by PI3-K inhibitors; however, the inhibition of MAP kinase activation did not directly correlate with the ability of these inhibitors to inhibit PI3-K activity, suggesting that enzymes other than PI3-K, that function upstream of MAP kinase, may be inhibited instead (Scheid and Duronio, 1996). However, expression of dominant negative PI3-K mutants has also been shown to affect activation of MAP kinase. Erk2 activation in response to T cell receptor engagement was inhibited by overexpressing a mutated form of p85 (Von Willebrand *et al.*, 1996; Jascur *et al.*, 1997). This p85 mutant lacks the iSH2 p110 binding site and has previously been shown to act in a dominant negative manner by blocking catalytic activation of the p110 subunit (Hara *et al.*, 1994). In addition, adenovirus-mediated transfer of the p85N-SH2 domain inhibits activation of erk1 and erk2 in response to insulin in 3T3-L1 adipocytes, despite elevation in GTP-bound Ras, suggesting that PI3-K may be required for activation of MAP kinase at a step independent of and downstream of Ras (Sharma *et al.*, 1998).

### **I.H.3 The Jak-STAT pathway**

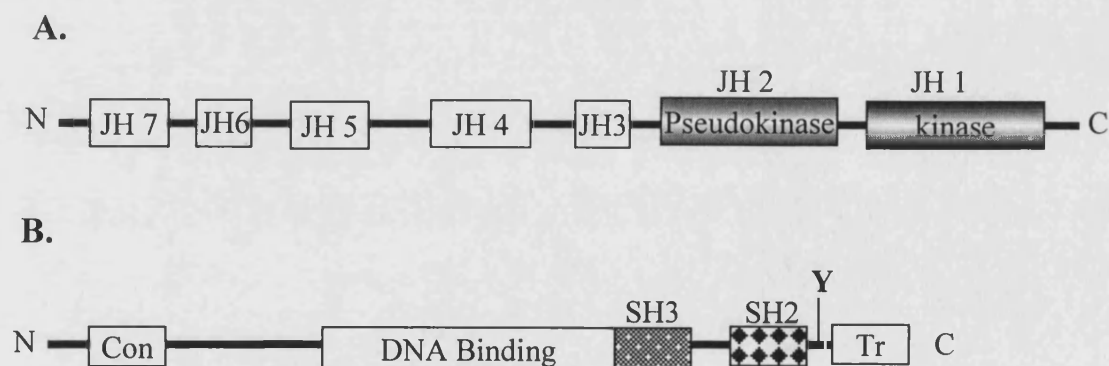
#### **I.H.3a The Janus kinase (Jak) family**

The Jak family of kinases in mammalian cells consists of Jak1, Jak2, Jak3, and Tyk2 (reviewed by Schindler and Darnell, 1995). This family was identified independently by low stringency hybridisation (Firmnach-Kraft *et al.*, 1990) and a polymerase chain reaction (PCR) approach (Wilks *et al.*, 1989; Partanen *et al.*, 1990) designed to identify

novel protein tyrosine kinases. Jak was originally an acronym for just another kinase, but it has also been proposed as an acronym for Janus (the Roman God of gates and doorways) kinase. Jak1, Jak2 and Tyk2 are expressed ubiquitously in many tissues whereas Jak3 appears to be predominantly expressed in myeloid cells, natural killer cells and activated T lymphocytes (Rane *et al.*, 1994; Johnston *et al.*, 1994).

Jak kinases share an overall structural pattern with seven conserved domains designated JH segments JH1 to JH7 (see Fig. I.13 A). Notable features include the absence of SH2 and SH3 domains and the presence of two tandem tyrosine-kinase domains. Only the most carboxy-terminal kinase domain, JH1, is believed to be functional, containing all the motifs associated with protein tyrosine kinases (Hanks *et al.*, 1988). The pseudo kinase domain JH2, immediately amino-terminal to JH1, contains kinase motifs but some of these lack several residues that are essential for catalytic activity and its function is yet to be established.

**FIGURE I.13**



### Structure of Jaks and STATs.

(A) The overall organisation of Jak family members. The seven conserved domains, designated JH 1 to JH 7 are indicated. The carboxy-terminal kinase domain, JH 1, is the functional kinase domain. The pseudokinase domain, JH 2, lies immediately amino-terminal to JH 1. (B) The functional domains of the STATs are indicated, including a conserved region in the amino terminus (Con), the DNA-binding domain, a SH3-like region (SH3), the highly conserved SH2 domain (SH2), the critical site of tyrosine phosphorylation (Y), and the carboxy-terminal transcriptional activation domain (Tr).

In general, Jak kinases are catalytically inactive in resting cells but are associated with the cytoplasmic domains of cytokine receptors. They are rapidly activated by a ligand-stimulated phosphorylation on a tyrosine within the kinase domain and all cytokines which interact with the type I cytokine receptor family activate member(s) of the Jak kinase and STAT proteins. Jak2 is phosphorylated and its kinase activity stimulated in response to many cytokines including GM-CSF (Quelle *et al.*, 1994), IL-3 (Silvennoinen *et al.*, 1993), Epo (Witthuhn *et al.*, 1993), IL-6 (Narazaki *et al.*, 1994) and growth hormone (Artgetsinger *et al.*, 1993). Several lines of evidence using receptor mutants have shown that box1 (see section I.E)-a conserved motif of the cytokine receptor superfamily located in a membrane proximal region- is required for activation and interaction of Jak2 with  $\beta c$  (Quelle *et al.*, 1994), growth hormone receptor, Epo receptor, and gp130 (Witthuhn *et al.*, 1993; Tanner *et al.*, 1995). Jak2 is known to associate with the GM-CSF receptor  $\beta c$  chain through its amino-terminal domain (Zhao *et al.*, 1995). In the case of receptors that contain single chains (Epo, growth hormone, prolactin and G-CSF), Jak is activated by receptor aggregation which induces its own trans-phosphorylation. Thus, after ligand stimulation, dimerisation of cytokine receptor subunits follows, which then induces the dimerisation of associated Jaks and results in cross-phosphorylation of the autophosphorylation site, resulting in activation of kinase activity. A similar mechanism is envisioned for receptors with multiple chains including those for IL-3 and GM-CSF. The mechanism by which Jak signals are downregulated involves the PTPase, SHP-1 (see section I.G.1a). Initially, it was shown that Epo-induced activation of its receptor negatively regulates Jak2 by SHP-1, which binds to the phosphorylated tyrosine residue in the carboxy-terminal region of the receptor and subsequently dephosphorylates and inactivates Jak2 (Klingmuller *et al.*, 1995). A similar mechanism is also predicted for regulation of  $\beta c$ , but it remains to be proven whether or not activities of all Jak family members are regulated negatively by SHP-1.

In cytokine receptor systems, the function of Jak in signalling is not limited to STAT activation and is believed to be responsible for the tyrosine phosphorylation of cytokine receptors as well as of several SH2-containing signalling molecules. Phosphorylation of receptor tyrosine residue(s) by Jak2 allows SH2-containing signalling molecules, including STAT and adaptor proteins, to bind to the receptor. Experiments carried out using dominant negative Jak2 revealed that Jak2 mediates tyrosine

phosphorylation of  $\beta c$  in response to GM-CSF (Watanabe *et al.*, 1996). In addition, it was shown that activation of *c-fos* and *c-myc* promoters and cell proliferation induced by GM-CSF were mediated by Jak2 (Watanabe *et al.*, 1996). Jak1 and Jak2 have also been shown to associate with and phosphorylate SHP-2 (Yin *et al.*, 1997). Thus, it is likely that Jak2 is responsible for tyrosine phosphorylation of  $\beta c$ , phosphorylation of SH2 containing proteins, including SHP-2, and for *c-fos* activation through the MAP kinase cascade in response to IL-3.

### I.H.3b Signal Transducers and Activators of Transcription (STATs)

These proteins were recognised for their dual functions in signal transduction in the cytoplasm and activation of transcription in the nucleus, hence the name STAT. To date, six members of the STAT family with similar structural features have been identified (reviewed by Ihle, 1996) and each functions in the signalling pathways of specific cytokines. STAT proteins vary in size from 734 to 851 amino acids with the principle differences occurring at the carboxy-terminus. They are composed of an amino-terminal DNA-binding domain, and an SH3-like region and an SH2 domain located in the carboxy-terminal end (Ihle, 1996) (see Fig. I.13 B). The SH3-like region may play a role in binding to proline rich motifs but as yet has an undetermined role. The SH2 domain is the most highly conserved region and is virtually identical to the core SH2 domain of src. The SH2 domain plays three important roles. It is critical for the recruitment of STATs to the activated receptor complexes. It is required for the interaction with Jaks, which phosphorylate the STATs. Finally, the SH2 domain is required for STAT dimerisation and the associated ability to bind DNA (Ihle, 1996). There is also a conserved tyrosine residue within the carboxy-terminal region that is essential for dimerisation of STAT proteins and a conserved serine residue, the phosphorylation of which is essential for maximal trans-activation. Thus, phosphorylation of a cytokine receptor provides a binding site for the SH2 domain of STAT. The recruited STAT is phosphorylated at its carboxy-terminal tyrosine by Jak and can then dimerise through the phosphorylated tyrosine residue and the SH2 domain of another STAT protein and is further phosphorylated at a carboxy-terminal serine residue. The dimerised STAT proteins then translocate to the nucleus where they bind to DNA sequences, most of which are related to the gamma interferon activated site (GAS), a regulatory element in the promoter of IFN- $\gamma$ -inducible genes (Darnell *et al.*,

1994). It is not known whether or not STAT proteins are involved in cytokine-induced cell proliferation. However, using dominant negative carboxy-terminal truncated STAT 5, partial suppression of IL-3-induced proliferation is observed (Mui *et al.*, 1996).

IL-3, GM-CSF, and IL-5 have been shown to activate STAT 5 (Mui *et al.*, 1995). In the case of STAT 5, several isoforms have been identified. Two variants occur in mice, which arise from distinct genes (Mui *et al.*, 1995; Azam *et al.*, 1995). These closely related isoforms of STAT 5, termed STAT 5a and STAT 5b, have been shown to be highly homologous (>90% identical) at the protein level, varying mainly in their carboxy-terminal regions. Both molecules are ubiquitously expressed at comparable levels in all tissues examined and appear to be activated equally by IL-3, GM-CSF, and IL-5 (Mui *et al.*, 1995). These two proteins can either homo or heterodimerise, thus acquiring the ability to bind to specific DNA sequences (Mui *et al.*, 1995). STAT 5 activation has been shown to result in the induction of many genes, including *c-fos* (Mui *et al.*, 1996; Watanabe *et al.*, 1996). The study by Mui *et al.*, (1996) showed that IL-3 stimulation of Ba/F3 cells induced to express a dominant negative STAT 5, constructed by carboxy-terminal truncation, resulted in a significant reduction in the expression of *cis*, *pim-1*, *osm*, *Id-1* and *c-fos*, suggesting that these five genes are regulated by STAT 5-dependent pathways. Importantly, overexpression of wild-type STAT 5 restored the gene induction pattern. The sensitivity of *cis*, *pim-1*, *osm*, and *Id-1* to expression of dominant negative STAT 5 is consistent with studies which mapped the region of IL-3/GM-CSF receptor  $\beta$  chain responsible for their induction (Sato *et al.*, 1993) to the same membrane proximal region required for Jak2 (Quelle *et al.*, 1994) and STAT 5 (Mui *et al.*, 1995) activation. In contrast, although this membrane proximal domain is also responsible for *c-myc* induction, *c-myc* levels were not affected by expression of dominant negative STAT 5, dissociating *c-myc* induction from STAT 5 activation (Mui *et al.*, 1996). However, as *c-myc* induction is sensitive to tyrosine kinase inhibitors (Kinoshita *et al.*, 1995), this suggests that *c-myc* is induced by either a tyrosine kinase distinct from Jak2, or alternately, through a Jak2-dependent pathway which is distinct from that responsible for STAT 5 activation.



## **I.I Overview of IL-3 signal transduction**

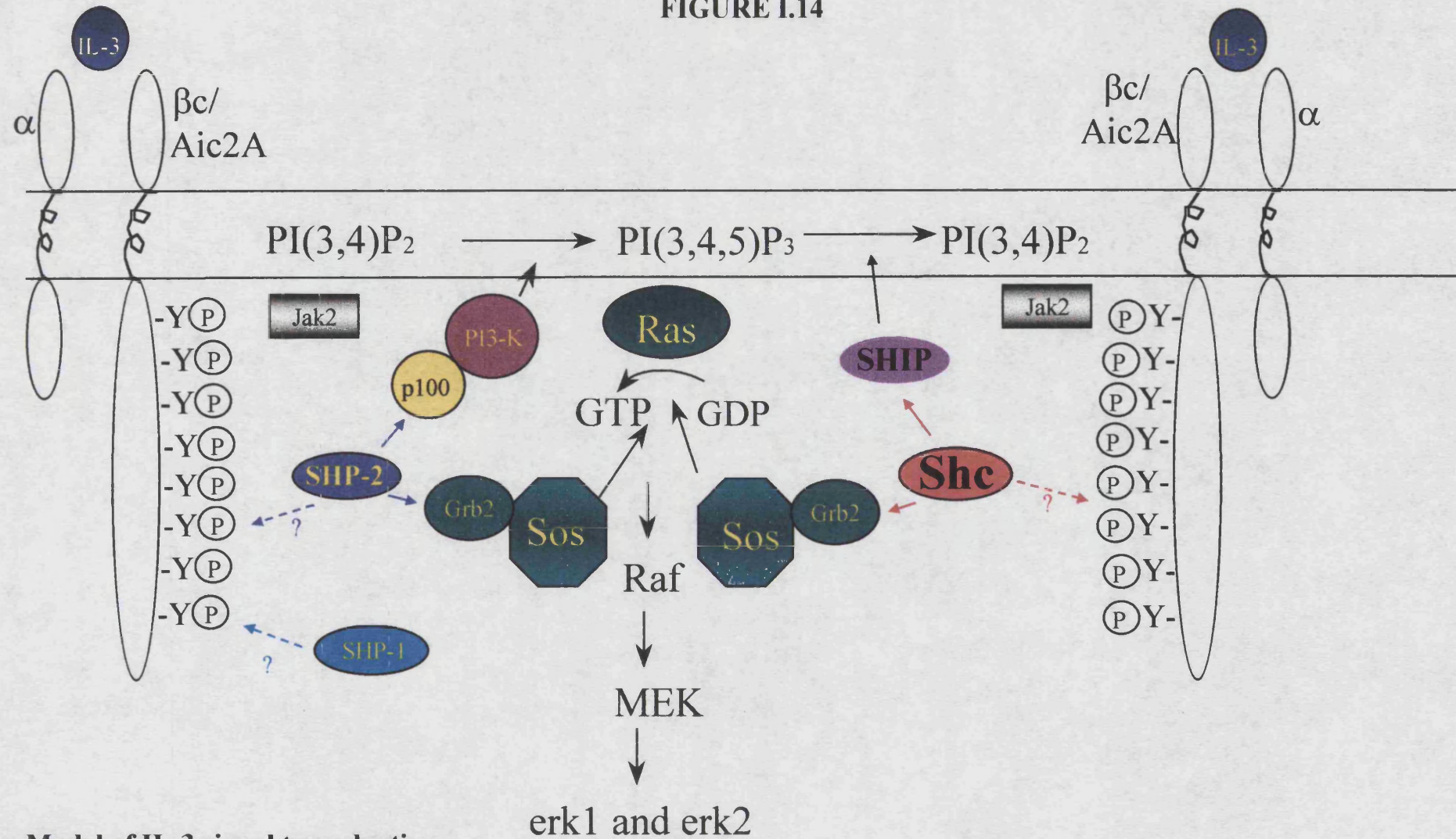
### **I.I.1 General**

IL-3, produced by activated T lymphocytes and mast cells, is a pleiotropic cytokine that acts as a potent growth factor for mast and other myeloid progenitor cells (Ihle, 1992). IL-3 has also been shown to be important for basophil and mast cell mediated immunity to parasites (Lantz *et al.*, 1998). Therefore, IL-3 plays an important role in mediating immune and inflammatory responses. The IL-3 receptor is composed of a 70 kDa specific  $\alpha$  chain and a 125-135 kDa  $\beta$  chain, both of which are members of the cytokine receptor superfamily. The human IL-3R  $\beta$  subunit is shared with the GM-CSF and IL-5 receptors and is termed  $\beta_c$ . Two different  $\beta$  subunits, Aic2A and Aic2B, exist in mice but Aic2A is specific for IL-3. Neither of the subunits of the IL-3R possess intrinsic tyrosine kinase activity. However, IL-3 has been shown to induce activation of both Jak2 (Silvennoinen *et al.*, 1993) and Src family (Anderson and Jorgensen, 1995) tyrosine kinases which correlate with the rapid tyrosine phosphorylation of a number of cellular proteins including: p42<sup>erk2</sup> and p44<sup>erk1</sup> (Welham *et al.*, 1992), p120 Jak2 (Silvennoinen *et al.*, 1993), p90 STAT 5 (Mui *et al.*, 1995), p70 SHP-2 (Welham *et al.*, 1994b), p46 and p52 Shc (Welham *et al.*, 1994a), p145 SHIP (Damen *et al.*, 1996) and the  $\beta$  subunit of the receptor itself (Sakamaki *et al.*, 1992; Duronio *et al.*, 1992a). This results in the activation of intracellular signalling cascades, including the Ras/MAP kinase and PI3-K pathways, and leading to cellular proliferation and survival.

### **I.I.2 Involvement of SHP-1 and SHP-2 in IL-3 signalling**

Phosphorylation of the IL-3 receptor  $\beta$  subunit, which contains 8 potential intracellular tyrosine phosphorylation sites, provides possible binding sites for molecules containing SH2 and PTB domains such as SHP-1, SHP-2 and Shc (see Fig. I.14). SHP-1 appears to act as a negative regulator of IL-3 signalling, as increased SHP-1 levels have been shown to suppress cell growth in response to IL-3 (Yi *et al.*, 1993). This may be accomplished through association with the IL-3 receptor as SHP-1 was shown to associate with the murine IL-3 receptor  $\beta$  subunit, Aic2A, although the sites of interaction were unknown (Yi *et al.*, 1993). SHP-2 is thought to act as a positive regulator of growth factor signalling. SHP-2 becomes tyrosine phosphorylated in response to IL-3 (Welham *et al.*,

FIGURE I.14



Model of IL-3 signal transduction.

1994b). This phosphorylation of SHP-2, on Tyr 304 and Tyr 542, creates docking sites for the SH2 domain of Grb2 (Welham *et al.*, 1994b). Thus, SHP-2 may function as an adaptor protein, localising the Grb2-Sos complex to the receptor and so to the vicinity of the plasma membrane associated Ras. In addition to SHP-2's possible role in the regulation of the Ras-MAP kinase pathway, SHP-2 has also been shown to co-precipitate with PI3-K following IL-3 stimulation (Welham *et al.*, 1994b). This association has been shown to be mediated by a 100 kDa protein which directly interacts with both the p85 subunit of PI3-K and SHP-2 in response to IL-3 (Craddock and Welham, 1997). Thus, SHP-2 may also function in localising the p100:PI3-K complex to the receptor and hence to the vicinity of the plasma membrane. This p100 protein has now been cloned and is known as Gab2 (Gu *et al.*, 1998). However, although SHP-2 has been shown to associate, via its SH2 domains, to the activated PDGFR, at tyrosine 1009 (Lechleider *et al.*, 1993b; Kazlauskas *et al.*, 1993), the EpoR at tyrosine 425 (Tauchi *et al.*, 1996), and to the EGFR (Feng *et al.*, 1993; Vogel *et al.*, 1993), no such association has been demonstrated with the IL-3 receptor in either murine or human cells.

### I.I.3 Involvement of Shc in IL-3 signalling

The p52 and p46 isoforms of Shc become highly tyrosine phosphorylated upon IL-3 stimulation of haemopoietic cells, correlating with activation of p21<sup>ras</sup> and the MAP kinases erk1 and erk2 (Welham *et al.*, 1994a). Functionally, Shc proteins have been implicated in regulating the activation of the Ras-MAP kinase pathway via a series of protein-protein interactions coupling the SH2 domain of Grb2 with tyrosine 317 of Shc, resulting in translocation of Shc-Grb2-Sos complex to the plasma membrane via interactions of Shc with activated receptors. Thus, Shc may be a key signalling molecule involved in the activation of erk kinases, which are themselves involved in the regulation of cell growth and differentiation (see Fig. I.14).

Shc has also been shown to associate with a 140-150 kDa inositol polyphosphate-5-phosphatase termed SHIP (Damen *et al.*, 1996). SHIP is tyrosine phosphorylated in response to IL-3 (Damen *et al.*, 1996; Lioubin *et al.*, 1996) and the association between SHIP and Shc is mediated by the interaction of both the SHIP SH2 domain binding directly to the motif surrounding tyrosine 317 of Shc, together with the NPXpY motifs of SHIP associating with the PTB domain of Shc, to form a high affinity Shc-SHIP complex (Liu *et*

*al.*, 1994; Liu *et al.*, 1997a). SHIP has been proposed to be a negative regulator of cell signalling because of its ability to dephosphorylate the primary *in vivo* product of PI3-K, phosphoinositol 3,4,5-P<sub>3</sub> (Lioubin *et al.*, 1996). Therefore, Shc may also play an important role in localising SHIP to the plasma membrane in response to IL-3.

With respect to localisation to the receptor, Shc has been shown to associate via its SH2 domain with  $\beta c$  following GM-CSF treatment, but the site of interaction was not determined (Lanfrancone *et al.*, 1995). Also, in a COS cell, non-ligand dependent system, Jak2 induced constitutive phosphorylation of  $\beta c$  tyrosine 577, which resulted in the binding of Shc to  $\beta c$  via its PTB domain (Pratt *et al.*, 1996). The association of Shc with  $\beta c$  in response to IL-3 has not been investigated. Interestingly though, upon IL-3 stimulation, no mass translocation of Shc to the plasma membrane has been observed as was after EGF stimulation (Ruff-Jamison *et al.*, 1993) and only a small increase in tyrosine phosphorylated Shc at the plasma membrane has been detected (Welham *et al.*, 1994a). Therefore, in response to IL-3, Shc may not simply function as an adaptor molecule, localising signalling molecules to the membrane, and may have alternative roles in the cytoplasm.

### **I.J Rationale and Specific Aims**

Upon stimulation of haemopoietic cells with IL-3, the PTPase SHP-2 and the adaptor protein Shc become highly tyrosine phosphorylated, suggesting an important role for these signalling molecules in mediating IL-3 signalling events, ultimately leading to cell growth, survival and differentiation. Both the SH2 domain containing PTPases, SHP-1 and SHP-2, have been implicated in IL-3 signalling. SHP-1 has been implicated as a negative regulator of IL-3-induced growth responses and has been shown to associate with the murine IL-3 receptor  $\beta$  subunit, Aic2A, although the sites of interaction were not mapped (Yi *et al.*, 1993). SHP-2 is thought to act as a positive regulator of growth factor signalling, possibly by acting as an adaptor molecule, localising the Grb2-Sos complex (Welham *et al.*, 1994b) and/or PI3-K (Craddock and Welham, 1997) to the plasma membrane. However, the association of SHP-2 with the IL-3 receptor, facilitating localisation of these signalling molecules to the membrane, has not previously been investigated.

Shc has been implicated as a key component in the regulation of the Ras/MAP kinase pathway. Tyrosine phosphorylation of Shc at position 317 creates a binding site for the SH2 domain of Grb2. Thus, the binding of Shc to activated receptors, results in localisation of the Grb2-Sos complex to the plasma membrane in the vicinity of Ras. Activation of Ras results in activation of a signalling cascade, ultimately leading to activation of the erk kinases which are involved in regulation of cell growth and differentiation. Shc has also been shown to associate with the inositol polyphosphate-5-phosphatase, SHIP, which has been proposed as a negative regulator of cell signalling because of its ability to dephosphorylate the primary *in vivo* product of PI3-K, PI(3,4,5)P<sub>3</sub>. The importance of Shc in regulating these signalling pathways relies on the association of Shc with the activated IL-3 receptor, yet this has not previously been studied.

Therefore, using a combination of biochemical, cellular and genetic techniques, this study attempted to investigate the roles of SHP-1, SHP-2 and Shc in haemopoietic cells in response to IL-3. Specifically, the protein-protein interactions mediated by SHP-1, SHP-2 and Shc in response to IL-3 were investigated, with an emphasis placed on interactions of these signalling molecules with the IL-3 receptor itself. The functional significance of the interactions of the various domains of Shc in integrating IL-3-mediated signalling events were explored further. How these interactions mediate IL-3 signal transduction with respect to activation of the Ras/MAP kinase pathway and cell proliferation was addressed.

## **CHAPTER II**

### **Materials and Methods**

## **II.A. Molecular Biology Techniques.**

### **II.A.1. Phenol/Chloroform extractions**

The supernatant containing the DNA was first extracted with an equal volume of buffer-saturated phenol:chloroform (1:1) and centrifuged for 1 minute at full speed in a Heraeus microfuge. The aqueous phase was then transferred to a clean tube and re-extracted with an equal volume of chloroform. After centrifugation for 1 minute, the aqueous phase was transferred to a clean tube, ready for ethanol precipitation.

### **II.A.2. Ethanol precipitation of DNA**

To the solution containing the DNA to be precipitated, 0.1 volume of 3 M sodium acetate, pH 5.5, and 2 volumes 100% ethanol was added. The solution was mixed by inversion, cooled on ice for 1-5 minutes and the precipitated DNA pelleted in a Heraeus centrifuge at 4°C for 10 minutes at full speed. For precipitation of some DNA fragments, the solution was first incubated in a dry ice/ethanol bath prior to pelleting. The ethanol was aspirated off and the pellet washed once in 70% ethanol. The pellet was dried briefly under a vacuum or left to air dry at room temperature. The pellet was then resuspended in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0) or water and stored at -20°C

### **II.A.3. Preparation of competent *E. coli***

A frozen stock of XL-1BL (Stratagene) was streaked onto a 2 x YT agar plate (16 g bacto-tryptone, 10 g yeast extract, 10 g NaCl, 15 g bacto-agar, adjusted to pH 7.5 with potassium hydroxide and made up to 1 L), inverted and incubated overnight at 37°C. A single colony was then used to inoculate 5 ml 2 x YT broth (same as 2 x YT agar, without the bacto-agar) which was then incubated overnight at 37°C in a shaking incubator. The following day, the bacteria were subcultured 1:100 in 100 ml 2 x YT broth and grown until an OD<sub>550</sub> of 0.48 was reached. The culture was then chilled on ice for 5 minutes and centrifuged in Beckman M5 centrifuge with a JA-14 rotor at 5000 rpm at 4°C for 10 minutes. The supernatant was removed and the bacteria resuspended in 0.4 volume (40 ml) of TfbI (30 mM KCl, 100 mM RbCl, 10 mM CaCl<sub>2</sub>, 50 mM MnCl<sub>2</sub>, 15% (v/v) glycerol, adjusted to pH 5.8 with 0.2 M acetic acid and filter sterilised), incubated on ice for 5 minutes and centrifuged at 5000 rpm for 5 minutes at 4°C. The sedimented bacteria were

then resuspended in 0.4 volume (5 ml) cold TfbII ( 10 mM MOPS, 75 mM CaCl<sub>2</sub>, 10 mM RbCl, 15% (v/v) glycerol, adjusted to pH 6.5 with KOH and filter sterilised) and incubated on ice for 15 minutes. Aliquots of 200 µl were snap frozen in 1.5 ml eppendorf tubes placed in a dry ice/ethanol bath and stored at -80°C.

#### **II.A.4. Transformation of *E. coli***

Competent cells were thawed at room temperature and placed on ice for 10 minutes. DNA was added at a concentration of < 100 ng/ 200 µl cells, mixed gently and left on ice for 30-45 minutes. The cells were heat shocked at 37°C for 2 minutes, returned to ice for 2 minutes and 4 volumes 2 x YT broth added. The cells were incubated for 1 hour at 37°C and the appropriate volume plated out onto 2 x YT agar plates containing appropriate antibiotic (100 µg/ml ampicillin). When ligations were transformed, the whole transformation was plated out by first spinning down the cells and then resuspending them in 100 µl 2 x YT broth, all of which was plated; otherwise, 100 µl of the total transformation was plated. The plates were then inverted and incubated at 37°C overnight.

#### **II.A.5. Small scale plasmid preparation**

A single bacterial colony was used to inoculate 3 ml 2 x YT broth containing 100 µg/ml ampicillin. The culture was incubated overnight at 37°C with vigorous shaking. The following day, 1 ml of culture was removed into an eppendorf tube and cells pelleted for 1 minute at full speed in a Heraeus microfuge. The pellet was resuspended in 100 µl solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) to which 200 µl fresh solution II ( 0.2 M NaOH, 1% (w/v) SDS) was added and the suspension incubated on ice for 5 minutes. 150 µl cold solution III ( 3 M potassium acetate, 2 M acetic acid) was added, the samples vortexed and incubated on ice a further 5 minutes. The precipitate was sedimented by centrifugation at full speed for 10 minutes at 4°C in a Heraeus microfuge, after which the supernatant was removed and transferred to a clean tube. The supernatant was extracted with phenol and chloroform as described in section II.A.1 and the DNA ethanol precipitated as described in section II.A.2. The DNA was finally dissolved in 30 µl water and stored at -20°C.



### **II.A.6. Large scale plasmid preparation**

Large scale plasmid preparations were performed using the QIAGEN Plasmid Midi Preparation protocol (QIAGEN) which is based on a modified alkaline lysis procedure. Briefly, 100 ml 2 x YT broth with 100µg/ml ampicillin was inoculated with a culture carrying the appropriate plasmid and incubated overnight at 37°C with vigorous shaking. The bacteria were pelleted by centrifugation in a Beckman M5 centrifuge with a JA-14 rotor at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 4 ml buffer P1. 4 ml buffer P2 was then added, the solution mixed and incubated at room temperature for 5 minutes, after which 4 ml chilled buffer P3 was added, mixed and incubated on ice for 15 minutes. The majority of the precipitated material was removed by centrifugation in the JA-14 rotor at 4°C for 5 minutes at 5000 rpm. The supernatant was transferred to 12 ml Beckman tubes for a further centrifugation in the JA-20.1 rotor at 4°C for 30 minutes at 15 500 rpm. The supernatant was applied to a QIAGEN-tip 100 that had been equilibrated with 4 ml of the low salt buffer, QBT. The tip was washed 2 times with 10 ml buffer QC, a medium salt buffer used to remove RNA, proteins, dyes and low molecular weight impurities. The DNA was then eluted with 5 ml of the high salt buffer, QF. Finally, the DNA was concentrated and desalted by ethanol precipitation as described in section II.A.2.

### **II.A.7. Restriction enzyme digestion**

New England Biolabs recommendations were followed with respect to appropriate buffers for each of the enzymes. The 10 X concentrated buffers supplied were aliquoted and stored at -20°C. A typical reaction was carried out in a volume of 20 µl, containing 2 µl 10 x restriction buffer, 1 µl (2 mg/ml) RNase (for mini prep DNA), 1-5 µl (approx. 1-5 µg) DNA, and 1 µl restriction endonuclease, made up to 20 µl with water. The reaction was mixed gently by flicking the side of the tube and then centrifuged briefly before being incubated at 37°C (room temperature for SmaI digestions) for 1-4 hours or overnight for linearization with PvuI before transfections (section II.B.3).

### **II.A.8. Conversion of 5' protruding ends to blunt ends**

To the 20 µl restriction enzyme digestion (containing 1-5 µl DNA), 3 µl 10X Klenow buffer was added and the reaction made up to 30 µl with water. 1.5 µl each dNTPs

(2 mM) were added along with 2  $\mu$ l Klenow and incubated at room temperature for 15 minutes. The reaction was then terminated by the addition of 1  $\mu$ l 0.5 M EDTA and heat inactivated at 75 °C for 10 minutes prior to gel purification (section II.A.12) or phenol/chloroform extraction and ethanol precipitation (sections II.A.1 and II.A.2).

#### **II.A.9. Treatment of plasmid DNA with Calf Intestinal Phosphatase (CIP)**

The appropriate plasmid was restriction enzyme digested with the required enzyme(s) (section II.A.7). The reaction was then set up as follows: 10  $\mu$ l 10X low salt restriction enzyme buffer, the 20  $\mu$ l restriction digest, distilled water to 100  $\mu$ l and 1  $\mu$ l (10 U) Calf Intestinal Phosphatase (CIP). The reaction was incubated at 37 °C for 30 minutes. To stop the reaction, 1  $\mu$ l EDTA, pH 8.0 was added. The CIP was heat inactivated at 70 °C for 10 minutes. The solution was then phenol/chloroform extracted and ethanol precipitated (sections II.A.1 and II.A.2). The phosphatase treated plasmids were stored in distilled water at -20°C.

#### **II.A.10. Ligations**

Ligations were carried out in 10  $\mu$ l volumes, typically containing 1  $\mu$ l 10 x T4 DNA ligase buffer, 1-4  $\mu$ l DNA insert, 1-2  $\mu$ l DNA vector, distilled water to 9  $\mu$ l and 1  $\mu$ l T4 DNA ligase. The mix was briefly centrifuged in the Heraeus microfuge and ligations incubated overnight at room temperature.

#### **II.A.11. Agarose gel electrophoresis**

Agarose gel solutions were prepared by boiling the appropriate quantity of agarose (for 1-2 % (w/v) gels) in 1 x TAE buffer (50x stock: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0). Once cooled, the gel was cast on a gel tray with ends sealed in masking tape and comb positioned a few mm from the bottom. Once set, the comb and tape were removed and the gel and tray submerged in a tank containing 1 x TAE buffer. 1/6<sup>th</sup> volume of a 6x gel loading buffer (30% (v/v) glycerol, 0.05% (w/v) Xylene/Cyanol, 0.05% (w/v) bromophenol blue) was added to the samples, centrifuged briefly and loaded on the gel. Electrophoresis was performed at 80 V. The gel was then submerged in a solution of approx. 0.5 $\mu$ g/ml ethidium bromide in 1x TAE buffer for

approximately 10 minutes, visualised under ultra violet light and photographed using a Polaroid camera.

### **II.A.12. Gel purification of DNA fragments**

DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (QIAGEN). Briefly, restricted DNA was separated by agarose gel electrophoresis and the ethidium bromide stained gel visualised under ultraviolet light (sec. II.A.11). The required DNA fragment was excised from the agarose gel with a clean, sharp scalpel and transferred to an eppendorf tube. Approximately 800  $\mu$ l buffer QX1 was added to the gel slice and incubated at 50°C for approximately 10 minutes, until the agarose was completely dissolved. The sample was then loaded onto the QIAquick spin column and centrifuged for 1 minute at full speed in a Heraeus microfuge. The column was washed once in 0.75 ml buffer PE and centrifuged in the microfuge for 1 minute. Residual wash buffer was removed with a further 1 minute spin. The DNA was eluted with 30-50  $\mu$ l distilled water.

### **II.A.13. Polymerase chain reaction**

PCR reactions were carried out in 50  $\mu$ l volumes, containing 5  $\mu$ l 10 x Vent buffer, 1  $\mu$ l 10 mM dNTPs, 1  $\mu$ g sense oligonucleotide, 1  $\mu$ g antisense oligonucleotide, 1  $\mu$ l 100 mM MgSO<sub>4</sub>, 1  $\mu$ l template, made up to 48  $\mu$ l with sterile distilled water and 2  $\mu$ l Vent DNA polymerase. The reaction was carried out in Perkin Elmer GeneAmp PCR System 2400 for 25 cycles with a denaturation temperature of 94°C for 45 seconds, and annealing temperature of 47°C for 30 seconds and a polymerisation temperature of 72°C for 2 minutes.

## **II.B. Tissue Culture Techniques.**

### **II.B.1. Cell culture**

Mammalian cells were cultured in humidified incubators at 37°C, 5% CO<sub>2</sub> (v/v), in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum, 20  $\mu$ M  $\beta$ -mercaptoethanol, 100 units penicillin-streptomycin, and 2 mM glutamine. TF-1 is a human erythroleukemic cell line (Kitamura *et al.*, 1989) and was maintained in the above medium supplemented with 10% (v/v) gibbon IL-3 (gIL-3) conditioned medium derived from

AgX63/gIL-3 cells (section II.B.2). TF-1 cells respond to IL-3, GM-CSF, Epo, IL-4, IL-13 and insulin (Zurawski *et al.*, 1993; Kitamura *et al.*, 1989). Ba/F3 is a murine pro-B cell line that is dependent on IL-3 for proliferation (Palacios and Steinmetz, 1985). Ba/F3 cells were cultured in 5% JWW3 conditioned medium as a source of IL-3 (section II.B.2). Ba/F3 cells expressing the tetracycline transactivator (tTA) from the plasmid pUHD15-1, containing a puromycin selectable marker (Ba/F15-1) were a kind gift from Dr. A. Mui, DNAX, Palo Alto, California (Mui *et al.*, 1996). Ba/F15-1 cells were cultured as for Ba/F3 cells with the addition of 2 µg/ml tetracycline. Each week, all cells were passaged 1:5 in 5 x 5 ml falcon dishes.

### II.B.2. Preparation of conditioned media

AgX63/gIL-3 cells expressing gIL-3 or JWW3 cells expressing murine IL-3 (mIL-3) were cultured in 175 cm<sup>2</sup> tissue culture flasks for approximately 1 week, until the media began turning yellow. The media was then filtered through a glass fiber filter (Whatman) to remove cells and cell debris and then sterilised through a 0.2 µm bottle top filter (Nalgene). Sterilised conditioned media was stored in 200 ml bottles and frozen at -20 °C until required.

XTT dye reduction assays, as outlined in sec. II.B.5, were performed with Ba/F3 or TF-1 cells to test the JWW3 and gIL-3 conditioned media respectively. Briefly, a serial dilution series of conditioned media in RPMI 1640 was prepared across a 96 well tray. Washed cells were resuspended at  $1 \times 10^5$  cells per ml and 50 µl added per well (5000 cells). Cells were incubated for 72 hours at 37°C before developing with XTT. The concentration of conditioned media required for maximum growth was determined and for future work, the media was supplemented with that concentration of conditioned media: typically 5% JWW3 for Ba/F3 cells and 10% gIL-3 for TF-1 cells.

### II.B.3. Transfections

The DNA to be transfected, pUHD10-3neo vector containing cDNA of interest (see section III.C), was linearized overnight at 37°C (section II.A.7) in a 30 µl reaction containing 10 µg DNA and 3 µl PvuI. After digestion, the reaction was made up to 100 µl with water and the DNA purified by phenol/chloroform extraction (section II.A.1) and ethanol precipitation (section II.A.2). The DNA pellet was resuspended in 10 µl sterile

electroporation buffer ( 25 mM HEPES, pH 7.2, 140 mM KCl, 10 mM NaCl, 2 mM  $\text{MgCl}_2$ , 0.5% Ficoll 400, filtered through 0.2  $\mu\text{M}$ ). Ba/F315-1 cells were washed twice by resuspending in 10 ml electroporation buffer and then pelleting at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge. The washed cells were then resuspended in electroporation buffer at  $1 \times 10^7$  cells / 0.8 ml. In a sterile 0.4 cm electroporation cuvette (gap 50, BioRad), 0.8 ml cells plus 10  $\mu\text{l}$  (10  $\mu\text{g}$ ) DNA was added. Cells were electroporated in a BioRad Gene Pulser at 960  $\mu\text{F}$  and 450V. The cells were left to stand at room temperature for 20 minutes, before being plated out into 20 ml RPMI 1640 with 5% (v/v) JWW3 conditioned medium, and 1  $\mu\text{g/ml}$  tetracycline and incubated at 37°C. After 48 hours, a viable cell count, using trypan blue, was performed and cells made up at a concentration of  $5 \times 10^5$  viable cells / ml in RPMI 1640 with 5% (v/v) JWW3 conditioned medium, 2  $\mu\text{g/ml}$  tetracycline, 1.5  $\mu\text{g/ml}$  puromycin, and 1 mg/ml active G418. The cells were plated into 3, 96 well flat bottomed trays (Nunc) with 100  $\mu\text{l}$  ( $5 \times 10^4$  cells) per well. The remaining cells were plated into a falcon 100 x 20 mm dish as a polyclonal population. After approximately 10 days, G418 and puromycin resistant clones from the 96 well trays were apparent and picked into 1 ml of the same selective media in a 24 well tray (Nunc). Clones were then expanded and screened for inducible expression of the introduced cDNAs (section II.B.4). Each subsequent week, selected clones were passaged (section II.B.1) in RPMI 1640 with 5% JWW3 conditioned medium and 2  $\mu\text{g/ml}$  tetracycline only.

#### **II.B.4. Screening for tetracycline-regulated expression**

G418 and puromycin resistant clones were expanded in RPMI 1640 with 5% (v/v) JWW3 conditioned medium in the presence of 2  $\mu\text{g/ml}$  tetracycline. Cells were then washed twice by resuspending in 1 X Hanks buffered saline solution containing 20 mM HEPES and pelleting at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge, to remove all traces of tetracycline. The washed cells were resuspended at  $1 \times 10^5$  cells/ml in the presence or absence of 2  $\mu\text{g/ml}$  tetracycline. At various intervals, samples were subsequently removed, washed once in phosphate buffered saline (PBS) and cell extracts prepared at approximately  $5 \times 10^5$  cells per 50  $\mu\text{l}$  solubilisation buffer (section II.C.1). Bradford assays (section II.C.9) were performed on the cell extracts and 15  $\mu\text{g}$  of protein loaded into each well of the acrylamide gel (section II.C.6).

### **II.B.5. XTT dye reduction proliferation assay**

Recombinant mIL-3 dilutions were set up in Nunc flat bottomed 96 well trays in triplicate. In column 1, 100 µl of 4 ng/ml rmIL-3 (twice the desired starting concentration) in serum free AIM-V media was added with columns 2-12 containing 50 µl AIM-V media alone. A serial dilution series was prepared across the plate by removing 50 µl from wells in the first column and mixing gently with wells in the next column with a multichannel pipette, repeating to column 12. Cells expressing the various mutant Shc proteins were expanded in the presence of 2 µg/ml tetracycline to repress expression. Cells were then washed three times with 1 X Hanks buffered saline solution containing 20 mM HEPES and resuspended at  $2 \times 10^4$  cells per ml in AIM-V media in the absence or presence of 2 µg/ml tetracycline. 50 µl of cells (1000 cells) were then added per well. Cells were incubated for 72 hours at 37°C. To harvest the assay, 25 µl of a solution containing 1 mg/ml XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulphonic acid hydrate) and 25 µM phenazine methosulphate (PMS acts as an electron-coupling reagent and is used to potentiate XTT bio-reduction) was added per well and incubated at 37°C for 4 hours (Roehm *et al.*, 1991). The soluble formazan product was measured at 450 nm on a Dynatech MR5000 plate reader.

### **II.B.6. Cell storage**

Cells were pelleted at 1500 rpm for 5 minutes in a Jouan CR412 centrifuge. The cell pellet was resuspended in 90% (v/v) foetal calf serum and 10% (v/v) DMSO at a concentration of greater than  $2 \times 10^6$  cell / ml. 1 ml aliquots were transferred into 1 ml Nunc cryotubes, wrapped in tissue, placed in a polystyrene box and incubated at -80°C. After 24 hours, the vials were transferred to liquid nitrogen.

## **II.C. Protein Chemistry Techniques.**

### **II.C.1. Cell stimulation and growth factors**

Gibbon IL-3 expressed in AgX63 cells is fully bioactive on human cells and was used as a source of IL-3 for stimulation of TF-1 cells. Ba/F3 cells and Ba/F15-1

transfectants were stimulated with recombinant mIL-3. Prior to stimulation, Ba/F15-1 transfectants were washed twice in 1 X Hanks buffered saline containing 20 mM HEPES to remove the tetracycline, plated at  $1 \times 10^5$  cells per ml in 175 cm<sup>2</sup> flasks and incubated in the absence or presence of 2 µg/ml tetracycline for 16 hours to induce protein expression. All cells were washed 3 times in Hanks buffered saline solution and starved of serum and IL-3 by resuspending the cells at a concentration of  $1 \times 10^7$  cells/ml (TF-1) or  $2 \times 10^7$  cells/ml (Ba/F3 and Ba/F15-1 transfectants) in serum free RPMI 1640 + 20 mM HEPES and incubating in a 37°C water bath for 20 minutes (TF-1 cells) or 45 minutes (Ba/F3 cells and Ba/F15-1 transfectants). TF-1 cells were then stimulated with gIL-3 conditioned medium (33% (v/v) final concentration) for 10 minutes to induce maximal levels of tyrosine phosphorylation of cellular substrates. Unless otherwise stated, Ba/F3 and Ba/F15-1 transfectants were stimulated with 20 ng/ml recombinant mIL-3 for 10 minutes to induce maximal levels of tyrosine phosphorylated cellular substrates. After stimulation, all cells were then pelleted at 4°C in a Heraeus microfuge for 20 seconds, supernatant removed by aspiration, and cells solubilised at  $2 \times 10^7$  cells / ml in ice cold solubilisation buffer (50 mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40 (NP-40), 150 mM NaCl, 5 mM EDTA, 10 mM sodium fluoride, 40 µg/ml phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, and 0.7 µg/ml pepstatin). The solubilised cells were pelleted at 4°C for 2 minutes at full speed in a Heraeus microfuge to pellet cell debris and the supernatant transferred to a clean tube.

### II.C.2. Immunoprecipitations

Cell extracts, typically from the equivalent of  $1 \times 10^7$  cells, were incubated with antibody on ice for 30 minutes. The antibodies and quantity used are outlined in Table II.1. Protein A-Sepharose beads or Protein G-Sepharose beads (30 µl of a 50% (v/v) slurry) were then added to the immunoprecipitate and samples incubated at 4°C on a rotator for 1 hour. Immunoprecipitates were centrifuged for 1 minute at full speed in a Heraeus microfuge at 4°C and then washed 3 times by adding 1 ml solubilisation buffer (section II.C.1) and pelleting for 1 minute at full speed in a Heraeus microfuge at 4°C. After the final wash, the bound protein was eluted by boiling in 20 µl 1 x SDS-PAGE sample buffer (5x stock: 10% (w/v) SDS; 50% (v/v) glycerol; 0.2 M Tris-HCl, pH 6.8; 5% (v/v) β-mercaptoethanol; bromophenol blue to colour). The samples were boiled for 2-5 minutes

and the beads pelleted by centrifugation in a Heraeus microfuge for 1 minute. The entire 20  $\mu$ l sample was loaded onto an SDS-PAGE acrylamide gel (section II.C.6).

Precipitations using GST fusion proteins were performed in a similar manner. The following amounts of GST fusion protein were used per precipitation: 15  $\mu$ g SHP-1(SH2)<sub>2</sub>-GST, 5  $\mu$ g SHP-2(SH2)<sub>2</sub>-GST, 10  $\mu$ g Grb2SH2-GST, and 10  $\mu$ g/ml all Shc-GST fusion proteins. Bound proteins were extracted using 30  $\mu$ l (of a 50% (v/v) slurry) glutathione Sepharose.

### II.C.3. Bacterial expression and purification of GST fusion proteins

*Escherichia coli* strain XL-1BL (Stratagene) transformed (section II.A.4) with pGEX2T plasmid (Pharmacia Biotech) carrying the DNA of interest (section III.B) were grown overnight in 10 ml 2 x YT broth containing ampicillin (100  $\mu$ g/ml) at 37°C. The culture was then subcultured 1:50 in 500 ml 2 x YT with ampicillin and grown at 37°C to an OD<sub>600</sub> of 0.6-0.8. The culture was then induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) at 27°C overnight. The bacteria were harvested by centrifugation at 4 000 x g for 10 minutes at 4°C in a Beckman centrifuge with a JA-14 rotor and the sediment resuspended to 15 ml in buffer TBN<sub>150</sub> (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM  $\beta$ -mercaptoethanol) containing protease inhibitors (10  $\mu$ g/ml leupeptin, 1.4  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml soybean trypsin inhibitor, 40  $\mu$ g/ml PMSF, 5  $\mu$ g/ml aprotinin) on ice. The cells were lysed with lysozyme at a final concentration of 0.1 mg/ml for 10 minutes at 25°C and subsequently subjected to three cycles of alternating freeze-thaws in a dry ice/ethanol bath and 37°C water bath. The crude lysate was adjusted to 10 mM MgCl<sub>2</sub> and 50  $\mu$ g/ml DNase and incubated at 25°C for 15 minutes before EDTA, pH 8.0 and NP-40 were added to a final concentration of 20 mM and 2.5% (v/v), respectively, and extracts incubated for a further 15 minutes at 25°C. After centrifugation at 30 000 x g for 30 minutes at 4°C, in a Beckman centrifuge with a JA-20.1 rotor, the supernatant was immediately decanted into a clean falcon tube and frozen at -80°C in 1 ml aliquots (= crude extract). Protein was purified by incubating 1.0 ml of the crude extract with 500  $\mu$ l packed glutathione Sepharose beads at 4°C, rotating for 2 hours, after which the beads were added to a column. The beads were washed 1 x 10 ml PBS, 2 x 10 ml PBS + 0.5% NP40, and 1 x 10 ml PBS. The GST fusion proteins were then eluted off the column batchwise with 5 x



0.5 ml aliquots of 20 mM glutathione, pH 7.5 (for 5 ml: 0.0307g glutathione, 1.5 ml Tris-HCl, pH 7.5, 2.5 µl each of the protease inhibitors) with each addition left to incubate with the beads for 10-20 minutes prior to elution. The purified GST fusion proteins were dialysed over night in 1 L PBS at 4°C, the PBS being changed 3 times. Protein concentration was then determined by Bradford assay (section II.C.9).

#### II.C.4. Phosphopeptides

The synthesis, purification and mass spectroscopic analysis of the synthetic phosphopeptides corresponding to the tyrosine residues within  $\beta$ c was performed by Dr. Ian Clark-Lewis (Biomedical Research Centre, Vancouver, British Columbia, Canada) and has been described elsewhere (Dechert *et al.*, 1994; Harder *et al.*, 1994). Phosphopeptides corresponding to the tyrosine residues within Aic2A were synthesized by Alta Bioscience (University of Birmingham, Birmingham, U.K.). Table II.2 lists the phosphopeptides used in this study. Throughout the text, the phosphotyrosine-containing peptides are referred to by the relative position of the tyrosine residue in  $\beta$ c, lacking the 14 amino acid signal sequence and pY indicates the position of the phosphotyrosine.

#### II.C.5. Phosphopeptide competition assay

5-20 µg of the GST-fusion proteins were pre-incubated with 100 µM phosphopeptide (or the appropriate concentrations for titration experiments) and 30 µl (50% (v/v) slurry) glutathione Sepharose and rotated at 4°C for 60 minutes. Cell extracts from  $1 \times 10^7$  cells, also containing 100 µM (or the indicated concentration) of the relevant phosphopeptide, were then added to the preincubation mixture and rotated at 4°C for a further 60 minutes. The beads were then washed and eluted as described in section II.C.2.

#### II.C.6. SDS polyacrylamide gel electrophoresis

The BioRad mini-Protean II gel electrophoresis system was used and a procedure similar to that of Laemmli (1970) followed. Glass plates were cleaned in 70% ethanol and apparatus assembled as per manufacturers instructions with 1 mm spacers. The running gel was prepared at the desired percentage of acrylamide (for 15 ml at 7.5% (v/v): 3.75 ml 4 x lower gel buffer [1.5 mM Tris-HCl, pH 8.8, 0.4% (w/v) SDS], 3.75 ml 30% (w/v) acrylamide/bis solution, 37.5:1, 7.45 ml water), and 50 µl 10% (w/v) ammonium

persulphate, and 15  $\mu$ l TEMED were added to catalyze polymerization of the acrylamide. The separating gel was cast by pouring 4.5 ml of the acrylamide solution between the two plates, which was then overlaid with water and allowed to polymerised for approximately 15 minutes. After polymerisation, the water was removed, the stacking gel (for 7.5 ml at 5% (v/v) acrylamide: 1.875 ml 4 x upper gel buffer [ 0.5 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS], 1.25 ml 30% (w/v) acrylamide/bis solution, 37.5:1, 4.35 ml water, 50  $\mu$ l 10% (w/v) ammonium persulphate, and 15  $\mu$ l TEMED) poured on top and a 15 well, 1 mm comb inserted to form wells. After polymerisation the comb was removed and the wells washed with water and filled with 1 x SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS). Once the samples were loaded, the gels were set in a tank with 1 x SDS-PAGE running buffer in both the top and bottom reservoirs. The gels were then run at 80V through the stacking gel and 180V through the separating gel. When the dye had reached the bottom of the gel the gel was removed and placed in a box containing 1 x semi-dry transfer buffer (39 mM glycine, 48 mM Tris base, 0.0375% (w/v) SDS, 20% (v/v) methanol).

### II.C.7. Immunoblotting (“Western Blotting”)

Gels were transferred to nitrocellulose by semi-dry transfer on a Pharmacia LKB NovaBlot. Four pieces of 3 MM Whatman paper, cut to the size of the gel and dampened with semi-dry transfer buffer, were placed one on top of each other on the bottom (positive) graphite electrode, to form a sandwich. Buffer saturated nitrocellulose was then placed on top of the stack, followed by the gel. Four more 3 MM Whatman papers were built up on the stack and the upper electrode placed on top. The gel was transferred for 60 minutes at 0.8 mA per  $\text{cm}^2$ . After transfer, the membranes were stained with Ponceau S to check for even loading and to mark the molecular weight standards. The blots were then transferred to TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) to remove the Ponceau S stain and then incubated in blocking buffer ( 5% (w/v) BSA, 1% (w/v) ovalbumin, and 0.05% (w/v) sodium azide in TBS) overnight. Before addition of the primary antibody the blots were washed once in TBS. Primary antibodies were prepared in a 1:5 dilution of blocking buffer and used at the concentrations outlined in Table II.1. The blots were incubated for 3 hours with all primary antibodies except for the anti- $\beta$ c antibody and anti-phosphospecific p44/p42 MAPK antibody which were incubated overnight. The blots were then washed 1 x

10 minutes in TBS, 3 x 10 minutes in TBSN (TBS with 0.05% (v/v) NP-40), and 1 x 10 minutes in TBS. The blots were subsequently incubated for 1-2 hours with the secondary antibody. Both goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako, Dimension Laboratories, Mississauga, Ontario) were used at a concentration of 0.05 µg/ml (1:20 000 dilution) in TBSN. The secondary antibody was then washed from the blots as for the primary antibody. After the final washing, the blots were placed in a clean container and developed in ECL solution (Amersham), a chemiluminescent detection system, for 1 minute. Kodak XAR-5 film was used for detection of ECL signals. The films were scanned by a BioRad GS-670 imaging densitometer for presentation in this thesis.

#### **II.C.8. Stripping of immunoblots**

Blots were stripped completely of antibodies by incubation with stripping solution (62.5 mM Tris-HCl, pH 6.7, 2% (w/v) SDS, 100 mM β-mercaptoethanol) at 55°C for 60 minutes with periodic agitation. After extensive washing in TBSN, blots were reblocked prior to reprobing with antibody as described above (section II.C.7).

#### **II.C.9. Bradford protein estimations**

BSA of known concentration or samples of cell lysates were added to 0.5 ml aliquots of water in Eppendorf tubes. 0.5 ml of the Bradford reagent (200 mg Coomassie Blue G-250 in 200 ml 85% (v/v) H<sub>3</sub>PO<sub>4</sub>, made up to 1.0 L with water and then filtered) was then added to each tube and vortexed. 100 µl was then added to wells in a 96 well round bottom tray (Falcon) and the optical density at 595 nm was determined on a Dynatech MR5000 plate reader. A standard curve was constructed from the BSA standards and the protein concentrations of the cell lysates determined. Adapted from Bradford, 1976.

#### **II.C.10. Immune complex *in vitro* MAP kinase assays**

Cell extracts were prepared (section II.C.1) and immunoprecipitations carried out as described in section II.C.2 using extracts from the equivalent of 5 x 10<sup>6</sup> cells using 25 µl (50% (v/v) slurry) of either anti-erk1 or anti-erk2 agarose conjugated beads. Immunoprecipitates were washed two times in solubilisation buffer and once with kinase assay buffer (20 mM HEPES, 5mM MgCl<sub>2</sub>, 1mM EGTA, 5mM β-mercaptoethanol, 1mM

PMSF, 2 mM sodium orthovanadate, 10 µg/ml aprotinin). The bead pellet was then resuspended in 13 µl kinase assay buffer and 4 µl 5 mg/ml MBP (1 mg/ml final concentration). 5 µCi [ $\gamma$ - $^{32}$ P]ATP diluted in kinase assays buffer (so adding 3 µl to the reaction in a total volume of 20 µl) was then added to the tubes in 20 second intervals and incubated at 30°C for exactly 10 minutes. The reaction was stopped by adding 6 µl of hot 5 x SDS sample buffer (section II.C.2). After boiling for approximately 4 minutes, samples were centrifuged for 1 minute in a Jouan A14 centrifuge at maximum speed and the entire 20 µl reaction separated on a 15% acrylamide gel by SDS-PAGE (section II.C.6). After transferring to nitrocellulose and staining with Ponceau S, the top half of the blot was cut off and immunoblotted (section II.C.7) with anti-erk1 antibodies to show even precipitation of erk1 and erk2, and the bottom half was exposed to X-ray film to detect the incorporation of  $^{32}$ P into MBP.

**II.D. Materials.****II.D.1. Antibodies**

**TABLE II.1**  
**Antibodies used for blotting and precipitation.**

<b>Antibody</b>	<b>Source</b>	<b>Usage</b>
4G10 (monoclonal, anti-phosphotyrosine)	UBI, Lake Placid, New York, U.S.A. (cat. # 05-321)	Blot: 0.1 µg/ml
9E10 (monoclonal anti-myc-tag)	ATCC hybridoma line	Blot: 0.5 µg/ml I.P.: 5 µg/sample
anti-βc (monoclonal, 3D7)	Pharmingen, Cambridge Bioscience, Cambridge, U.K. (cat. # 18801D)	I.P.: 20 µg/sample
anti-βc (rabbit polyclonal, JS5)	gift of Dr. Vince Duronio, formerly of Biomedical Research Centre and now at Jack Bell Research Centre, Vancouver, B.C., Canada	Blot: 0.5 µg/ml
anti-erk1 (C-16) (rabbit polyclonal)	Santa Cruz (cat. # sc-93)	Blot: 0.1 µg/ml
anti-erk1 (C16) AC (rabbit polyclonal)	Santa Cruz (cat. # sc-93-G)	I.P.: 25 µl/sample
anti-erk2 (C-14) AC (rabbit polyclonal)	Santa Cruz (cat. # sc-154-G)	I.P.: 25 µl/sample
Anti-phosphospecific p44/p42 (Thr 202/Tyr 204) MAPK (rabbit polyclonal)	New England Biolabs (cat. # 9101S)	Blot: 1:2000
anti-p85(PI3K) (polyclonal rabbit antiserum, JS14.2)	Prepared by M.J. Welham at Biomedical Research Centre, Vancouver, B.C., Canada	I.P.: 2 µl
anti-Shc (rabbit anti-serum against the SH2 domain of Shc)	UBI, Lake Placid, New York, U.S.A. (cat. # 05-321)	Blot: 1:5000 I.P.: 2 µg/sample
anti-SHIP (5340)	Gift of Dr. L. Rohrschneider, Dept. of Pathology, University of Washington, Seattle, Washington, USA	Blot: 1:5000
anti-SHIP	Gift of Dr. K.M. Coggeshall, 911 Biosciences Building, 484 W. 12 <sup>th</sup> Avenue, Columbus, OH, USA	I.P.: 4 µl
anti-SHP1 (SHPTP1)	Santa Cruz (cat. # sc-287)	Blot: 0.5 µg/ml I.P.: 1 µg/sample
anti-SHP2 (SHPTP2)	Santa Cruz (cat. # sc-280)	Blot: 0.5 µg/ml I.P.: 0.4 µg/sample

## II.D.2 Phosphopeptides

TABLE II.2

Synthetic phosphopeptides corresponding to tyrosine residues within  $\beta c$  and Aic2A

Position of Y	Species	Sequence	Sequence of phosphopeptide
750	Human/ $\beta c$	KSGFEGpYVELPPI	EGpYVELP
745	Mouse/Aic2A	PPGFEDpYVELPPS	PGFEDpYVELP
695	Human/ $\beta c$	PGVASGpYVSSADL	SGpYVSSA
612	Human/ $\beta c$	PPGSLEpYLCLPAG	LEpYLCLP
610	Mouse/Aic2A	LPGSLEpYMCLPP	PGSLEpYMCLP
577	Human/ $\beta c$	FDFNGPpYLGPPHS	GPpYLGPP
575	Mouse/Aic2A	FDFNGPpYLGPPQS	FDFNGPpYLGPPQ
452	Human/ $\beta c$	FCGIYGpYRLRRKT	YGpYRLRR

## II.D.3. Reagents

TABLE II.3  
List of Reagents

Reagents	Supplier*
1 Kb DNA ladder	Life Technologies
$\beta$ -mercaptoethanol	Bio-Rad
[ $\gamma$ - $^{32}$ P] ATP	NEN
Acetic acid (glacial)	BDH
30% Acrylamide/Bis solution 37.5:1	Bio-Rad
agarose (electrophoresis grade)	Life Technologies
AIM-V	Life Technologies
Albumin, Chicken Egg (Grade III)	SIGMA
Ammonium persulphate	Fisons Scientific
Ampicillin	SIGMA
Aprotinin	Boehringer Mannheim
Bactoagar	Difco Laboratories
Bactotryptone	Difco Laboratories
Bovine Serum Albumin (BSA)	Boehringer Mannheim
Bromophenol Blue	Fisons Scientific
Calcium chloride ( $\text{CaCl}_2$ )	Fisons Scientific
Calf intestinal phosphatase (CIP)	New England Biolabs
Chloroform	BDH
Coomassie brilliant blue G-250	Bio-Rad
Diaminoethanetetra acetic acid (EDTA)	SIGMA
Dideoxy nucleotide triphosphates	New England Biolabs

(dNTPs)	
Dimethyl sulphoxide (DMSO)	SIGMA
DNase I	Boehringer Mannheim
ECL	Amersham
Ethanol (99.6%)	BDH
Fetal calf serum	Autogen Bioclear
Ficoll 400	SIGMA
Geneticin (G418)	Life Technologies
Glucose	Fisons Scientific
Glutathione	SIGMA
Glutathione Sepharose 4B	Pharmacia Biotech
Glycerol	SIGMA
Glycine	SIGMA
HANKS buffered saline (10x)	Life Technologies
HEPES	Life Technologies
IPTG	Life Technologies
Klenow	New England Biolabs
L-Glutamine (100X)	Life Technologies
Leupeptin	SIGMA
Lysozyme	SIGMA
Magnesium chloride (MgCl <sub>2</sub> )	SIGMA
Magnesium sulphate (50X)	New England Biolabs
Manganese chloride (MnCl <sub>2</sub> )	SIGMA
Methanol	BDH
MOPS	SIGMA
Myelin basic protein (MBP)	SIGMA
Nitrocellulose	BDH
NP-40	SIGMA
Oligonucleotides	Pharmacia Biotech
Penicillin-Streptomycin	Life Technologies
Pepstatin A	Boehringer Mannheim
Phenazine methosulphate (PMS)	SIGMA
Phenol (buffer-saturated)	Life Technologies
Phenylmethylsulphonyl fluoride (PMSF)	SIGMA
Phosphate Buffered Saline (PBS)	Life Technologies
Phosphoric Acid (85% (v/v)) H <sub>3</sub> PO <sub>4</sub>	SIGMA
Ponceau S	SIGMA
Potassium acetate (KAc)	SIGMA
Potassium Chloride (KCl)	SIGMA
Potassium Hydroxide (KOH)	SIGMA
Protein-A Sepharose	Pharmacia Biotech
Protein-G Sepharose	Pharmacia Biotech
Puromycin	Calbiochem
Recombinant mouse IL-3 (rmIL-3)	R&D Systems

Restriction Endonucleases	New England Biolabs
RNase A	Boehringer Mannheim
RPMI 1640	Life Technologies
Rubidium chloride	SIGMA
SDS-PAGE standards (broad range)	Bio-Rad
Sodium acetate (NaAc)	SIGMA
Sodium azide	Fisons Scientific
Sodium chloride (NaCl)	SIGMA
Sodium dodecyl sulphate (SDS)	BDH
Sodium hydroxide (NaOH)	Fisons Scientific
Sodium fluoride (NaF)	SIGMA
Sodium molybdate	BDH
Sodium orthovanadate	SIGMA
Soybean Trypsin Inhibitor	SIGMA
T4 DNA ligase	New England Biolabs
Tetracycline	SIGMA
Tetramethylethylenediamine (TEMED)	Bio-Rad
Trizma base (Tris)	SIGMA
Vent DNA polymerase	New England Biolabs
X-ray film (XAR-5)	Kodak
XTT	SIGMA
Xylene/Cyanol	SIGMA
Yeast extract	Difco Laboratories

**\*Full name and location of Suppliers:**

Amersham Pharmacia Biotech, Herts, U.K.

Autogen Bioclear, Wilts, U.K.

BDH Chemicals Ltd., Poole, U.K.

Bio-Rad, Richmond, California, USA

Boehringer Mannheim Ltd., East Sussex, U.K.

Calbiochem, Nottingham, U.K.

Difco Laboratories, Detroit, Michigan, USA

Eastman Kodak Company, Rochester, NY, USA

Fisons Scientific, Leisceseter, U.K.

Life Technologies Ltd., Paisley, U.K.

NEN Life Science Products, Holland

New England Biolabs Inc., MA, USA

R&D Systems Europe Ltd., U.K.

Sigma Chemicals, Poole, U.K.



## **CHAPTER III**

### **Protein-Protein interactions mediated by the protein tyrosine phosphatases (PTPases) SHP-1 and SHP-2**

### **III.A. Introduction and Aims.**

Relatively little is known about the action of IL-3 on protein tyrosine phosphatases. SHP-1 is expressed and constitutively tyrosine phosphorylated in haemopoietic cells and appears to negatively regulate IL-3-induced cell proliferation (Yi *et al.*, 1993). The related PTPase, SHP-2, is more ubiquitously expressed and is thought to act as a positive mediator of growth factor signals. It has been shown previously that IL-3 induces tyrosine phosphorylation of SHP-2, creating a docking site for the SH2 domain of Grb2 (Welham *et al.*, 1994b). In addition, IL-3 treatment of cells has been shown to result in the co-precipitation of phosphoinositol 3'-kinase (PI3-K) with SHP-2, as well as increasing the phosphatase activity of SHP-2 (Welham *et al.*, 1994b). By determining the protein-protein interactions mediated by SHP-1 and SHP-2 in response to IL-3, the possible roles for these PTPases in haemopoietic cells could be investigated.

### **III.B. Tyrosine phosphorylated $\beta$ c co-precipitates with SHP-1 and SHP-2 after IL-3 stimulation.**

A 135 kDa protein identified as the human IL-3 receptor  $\beta$  subunit ( $\beta$ c) and a 70 kDa protein identified as the PTPase SHP-2 (Welham *et al.*, 1994b), become tyrosine phosphorylated after IL-3 stimulation of haemopoietic cells. It has been demonstrated previously that SHP-2 can associate, via its SH2 domain, to activated receptors including the EGFR and PDGFR (Feng *et al.*, 1993; Vogel *et al.*, 1993; Lechleider *et al.*, 1993b). SHP-1 has been shown to associate with the murine IL-3R  $\beta$  subunit, Aic2A, via an unmapped site (Yi *et al.*, 1993) but no such association has been demonstrated in human cells for SHP-1 or SHP-2. Since SHP-2 had previously been shown to associate with Grb2 and co-precipitate with the p85 subunit of PI3-K (Welham *et al.*, 1994b), it was investigated whether SHP-1 and SHP-2 were able to bind to  $\beta$ c and thereby possibly function as adaptor molecules in IL-3 signalling. TF-1 cells were left untreated as a control (C) or stimulated with IL-3 (3). The cells were then lysed and the resulting extracts precipitated with either a monoclonal anti-IL-3 receptor  $\beta$  subunit antibody (anti- $\beta$ c) or polyclonal anti-SHP-1 or anti-SHP-2 antibodies. The

precipitates were separated by SDS-PAGE, transferred to nitrocellulose and subsequently blotted with 4G10 anti-phosphotyrosine antibodies. The results are shown in Figure III.1.

A tyrosine phosphorylated 135-140 kDa protein was immunoprecipitated with the anti- $\beta c$  monoclonal antibody after IL-3 stimulation (Fig. III.1 A). Reprobing this same blot with a polyclonal anti- $\beta c$  antibody confirmed this to be  $\beta c$  (Fig. III.1 B). The anti-SHP-1 antibodies precipitated three tyrosine-phosphorylated proteins of 135, 105, and 60 kDa (Fig. III.1 A) following IL-3 treatment. The 60 kDa phosphoprotein was present in SHP-1 precipitates from both control and IL-3-stimulated cells and blotting with anti-SHP-1 antibodies confirmed that this protein was SHP-1 (Fig. III.1 C). SHP-1 is constitutively phosphorylated in other haemopoietic cells (Yi *et al.*, 1993), and this also appears to be the case in TF-1 cells. The identity of the 105 kDa protein is unknown. The 135 kDa species co-migrated with the tyrosine phosphorylated  $\beta c$  precipitated by the anti- $\beta c$  antibodies in IL-3 stimulated cells (Fig. III.1 A), suggesting that tyrosine phosphorylated  $\beta c$  co-precipitates with SHP-1. Reprobing this same blot with polyclonal anti- $\beta c$  antibodies (Fig. III.1 B) could not confirm that this protein was indeed  $\beta c$ . This was probably because the amount of tyrosine phosphorylated 135 kDa species precipitated by the anti-SHP-1 antibodies was considerably less than that precipitated by the anti- $\beta c$  antibodies (Fig. III.1 A) and so below the limits of detection.

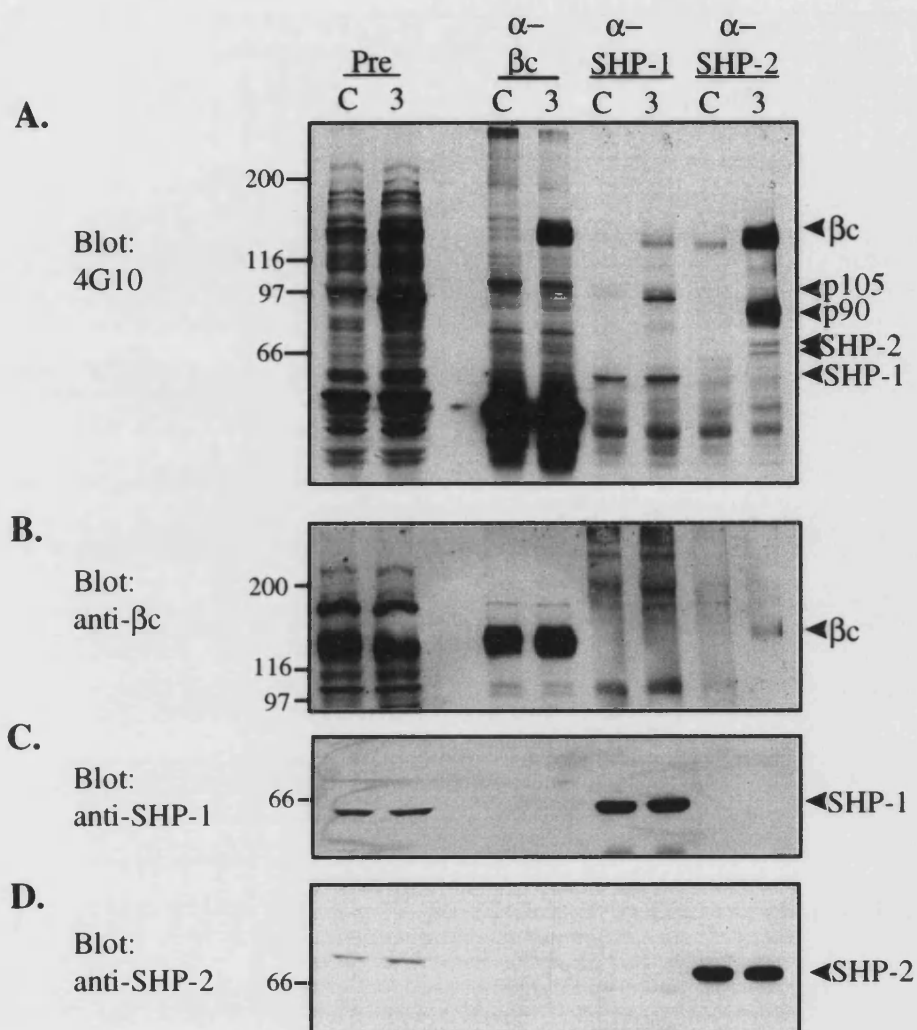
The SHP-2 antibodies precipitated four tyrosine phosphorylated proteins of 70, 72, 90, and 135 kDa from IL-3 stimulated TF-1 cells (Fig. III.1 A). The lower 70 kDa protein corresponds to SHP-2, as confirmed by immunoblotting with the SHP-2 antibody (Fig. III.1 D). The upper tyrosine phosphorylated 72 kDa protein is thought to be a hyper-phosphorylated form of SHP-2, but this was not formally demonstrated here. The identity of the broad 90 kDa protein is unknown. The 135 kDa protein co-migrated with tyrosine phosphorylated  $\beta c$  precipitated by the anti- $\beta c$  antibodies (Fig. III.1 A) and its identity as  $\beta c$  was confirmed by reprobing the blot with the polyclonal anti- $\beta c$  antibodies (Fig. III.1 B). In reciprocal experiments, in which blots of material precipitated by anti- $\beta c$  antibodies were reprobed with antibodies specific for either SHP-1 or SHP-2, neither were detectable. (Fig. III.1 C and D). The monoclonal antibody precipitates only 10-20 % of the  $\beta c$  expressed in TF-1 cells (M.J.

### FIGURE III.1

#### **SHP-1 and SHP-2 associate with tyrosine phosphorylated $\beta c$ after IL-3 stimulation.**

TF-1 cells were either left untreated as a control (C) or treated for 10 minutes with IL-3 (3).  $1 \times 10^7$  cells per sample were lysed and a sample of the lysate retained (Pre). The remaining lysates were incubated with either a monoclonal anti-IL-3R  $\beta c$  antibody, anti-SHP-1 antibodies, or anti-SHP-2 antibodies. Samples were separated through a 7.5% acrylamide gel by SDS-PAGE. (A) Immunoblotting was performed with 4G10 anti-phosphotyrosine antibodies. (B), the same blot as in A was stripped and reprobed with polyclonal anti- $\beta c$  antibodies. (C), the same blot as in B was stripped and reprobed with the polyclonal anti-SHP-1 antibodies. (D), the same blot as in C was stripped and reprobed with polyclonal anti-SHP-2 antibodies. The positions of  $\beta c$ , SHP-1 and SHP-2 are indicated. The molecular mass standards are shown and expressed in kDa. These data are representative of two separate experiments, with similar results observed in individual single immunoprecipitations from TF-1 cells from other experiments.

**FIGURE III.1**



Welham, unpublished data), only a portion of which is likely to be phosphorylated at the appropriate sites and will hence interact with downstream signalling molecules. Thus, the failure to detect SHP-1 and SHP-2 in the anti- $\beta$ c precipitates most likely reflects the fact that the amounts of SHP-1 and SHP-2 present were below the limits of detection. In addition, tyrosine phosphorylated proteins of SHP-1 and SHP-2 size were also not observed in the anti- $\beta$ c immunoprecipitates (Fig. III.1 A). However, these results do suggest that both SHP-1 and SHP-2 associate with the tyrosine phosphorylated  $\beta$ c after IL-3 stimulation.

### **III.C. SHP-1 and SHP-2 associate with $\beta$ c through their SH2 domains after IL-3 stimulation.**

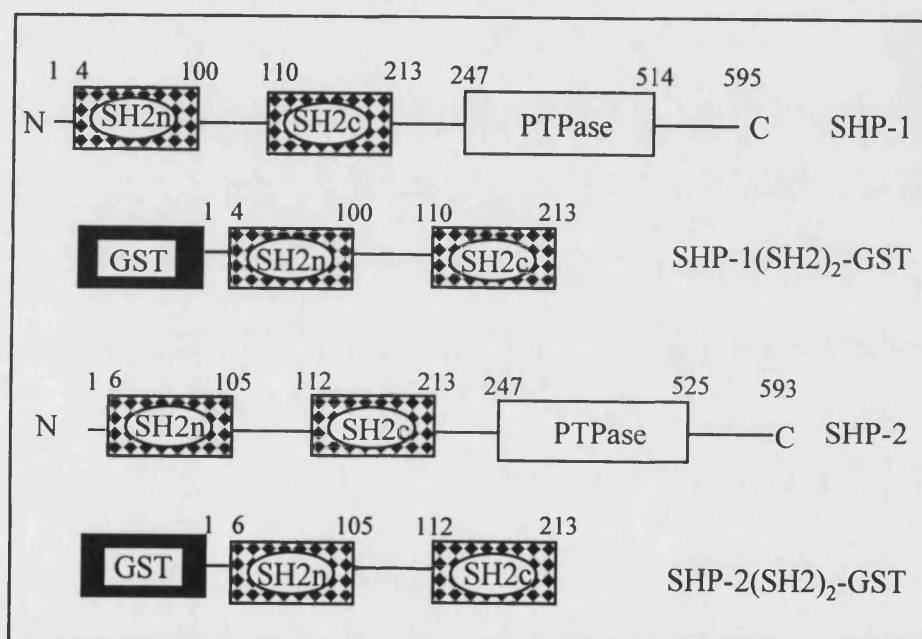
To determine whether the SH2 domains of the two PTPases directed their interaction with  $\beta$ c, fusion proteins containing either the SHP-1 SH2 domains or the SHP-2 SH2 domains fused to glutathione S transferase (GST) were used in precipitation analyses. The SHP-1 SH2 domains and the SHP-2 SH2 domains cloned into the pGEX2T vector (Pharmacia Biotech) were constructed by Ute Dechert (formerly of Biomedical Research Centre, Vancouver, B.C., Canada). These constructs were transformed into XL-1BL (section II.A.4) and large scale cultures were induced overnight with isopropyl  $\beta$ -thiogalactopyranoside (IPTG) and GST fusion proteins purified on glutathione Sepharose beads (section II.C.3). Figure III.2 depicts the structure of these GST fusion proteins.

The SHP-1(SH2)<sub>2</sub>-GST and SHP-2(SH2)<sub>2</sub>-GST fusion proteins were used for direct precipitation analyses from extracts of TF-1 cells that had either been left untreated as a control (C) or stimulated with IL-3 (3). The precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The results are shown in Figure III.3 A and are representative of three separate experiments. Both the SHP-1(SH2)<sub>2</sub>- and SHP-2(SH2)<sub>2</sub>- GST fusion proteins precipitated a tyrosine phosphorylated 135 kDa protein from extracts stimulated with IL-3 but not from control samples. This same blot was then stripped and reprobed with a polyclonal anti-  $\beta$ c antibody (Fig. III.3 A, lower panel) which reacted with the same tyrosine phosphorylated protein

precipitated by the SHP-1(SH2)<sub>2</sub>- and SHP-2(SH2)<sub>2</sub>- GST fusion proteins. These results clearly demonstrate that the SH2 domains of SHP-1 and SHP-2 associate *in vitro* with tyrosine phosphorylated  $\beta c$  following IL-3 stimulation.

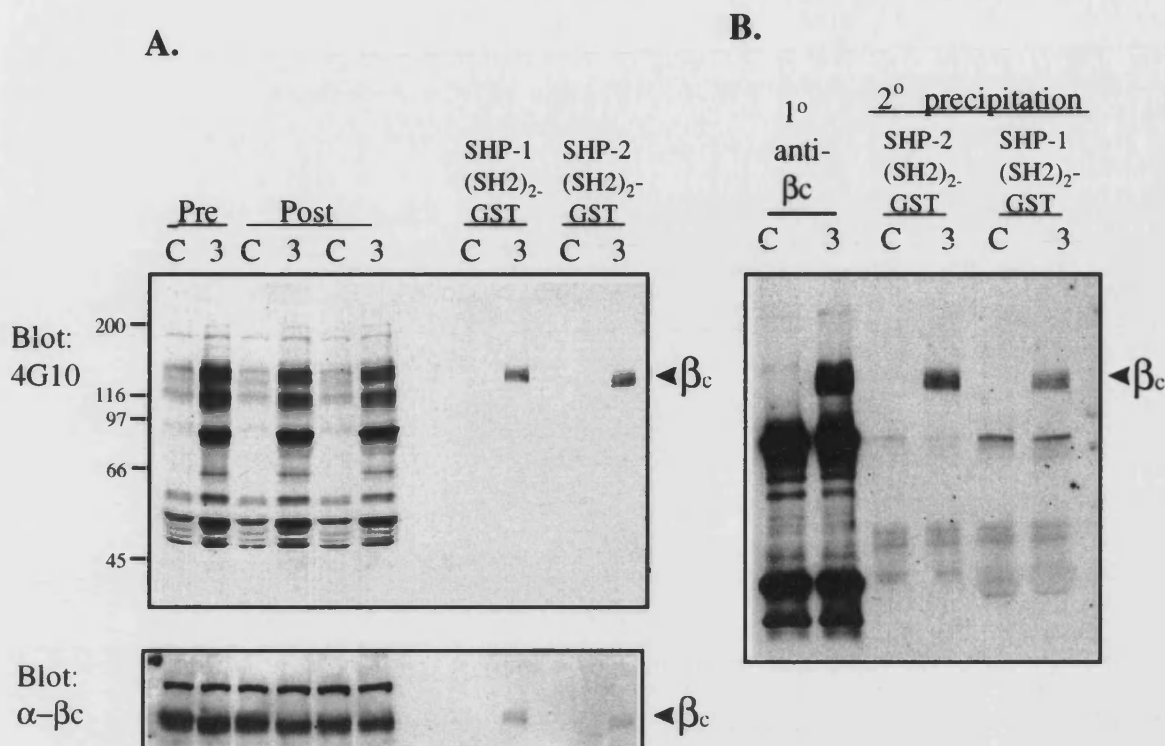
**FIGURE III.2**

**Schematic representation of the SH2 domains of SHP-1 and SHP-2 expressed as GST fusion proteins.**



To ascertain whether the interaction between  $\beta c$  and the SH2 domains of SHP-1 and SHP-2 were direct, sequential immunoprecipitations were performed. Sequential precipitations are used to determine whether the interaction between two proteins is direct *in vitro*. After boiling and denaturing a primary precipitation, the secondary precipitating agent is added, e.g., GST fusion proteins. Any re-precipitated proteins are likely to be a result of a direct interaction with the secondary agent since any intermediary molecule(s) will no longer be bound to the primary precipitated proteins (although formally renaturation could occur but would be unlikely). TF-1 cells were either stimulated with IL-3 (3) or left untreated as a

**FIGURE III.3**



**SHP-1 and SHP-2 directly associate with tyrosine phosphorylated β<sub>c</sub> through their SH2 domains.**

TF-1 cells were either left untreated as a control (C) or stimulated for 10 minutes with IL-3 (3). (A), cell extracts from the equivalent of  $1 \times 10^7$  cells per sample were precipitated using either SHP-1(SH2)<sub>2</sub>-GST or SHP-2(SH2)<sub>2</sub>-GST. "Pre" indicates samples removed prior to precipitation whereas "Post" indicates samples removed after precipitation. (B), cell extracts from the equivalent of  $4 \times 10^7$  cells per sample were precipitated with 20 μg of monoclonal anti-β<sub>c</sub> antibody. Primary anti-β<sub>c</sub> precipitates were eluted and denatured by boiling in SDS sample buffer and 1/10 of the sample saved for the primary anti-β<sub>c</sub> immunoprecipitation sample. Secondary precipitations were prepared with either SHP1(SH2)<sub>2</sub>-GST or SHP-2(SH2)<sub>2</sub>-GST. All samples were separated on 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The same blot in A was stripped and reprobed with polyclonal anti-β<sub>c</sub> antibodies (lower panel). The positions of the molecular mass standards are shown and expressed in kDa and the position of β<sub>c</sub> is indicated.



control (C). The extracts were immunoprecipitated first with monoclonal anti- $\beta c$  antibody (3D7) and after extensive washing the precipitated material boiled and denatured in the presence of SDS and  $\beta$ -mercaptoethanol, effectively denaturing any associated proteins. The resulting extracts were diluted 1:10 (so the concentration of SDS was  $<0.1\%$  (w/v)) and then re-precipitated with either the SHP-1(SH2)<sub>2</sub>- or SHP-2(SH2)<sub>2</sub>- GST fusion proteins. The results are shown in Figure III.3 B and are representative of two individual experiments. The primary anti- $\beta c$  immunoprecipitation precipitated tyrosine phosphorylated  $\beta c$  from cells treated with IL-3, but not from control cells as would be expected. The secondary precipitations with either the SHP-1(SH2)<sub>2</sub>- or the SHP-2(SH2)<sub>2</sub>- GST fusion proteins re-immunoprecipitated tyrosine phosphorylated  $\beta c$  from the IL-3 stimulated cell extracts. Similar experiments were also performed by first precipitating with the GST fusion proteins and then performing a secondary anti- $\beta c$  immunoprecipitation. Again, tyrosine phosphorylated  $\beta c$  was precipitated in both primary and secondary precipitations in IL-3 treated samples (results not shown). These results suggest that *in vitro* both SHP-1 and SHP-2 can bind directly to  $\beta c$  through their SH2 domains after IL-3 stimulation.

#### **III.D. Binding of SHP-1 and SHP-2 to $\beta c$ is inhibited by a phosphopeptide based on sequences surrounding tyrosine 612 of $\beta c$ .**

To confirm that the observed associations involved interactions of the SHP-1 and SHP-2 SH2 domains with phosphotyrosines on  $\beta c$  and to provide an indication as to which of the potential tyrosines on  $\beta c$  was responsible for mediating the interactions with the SH2 domains of SHP-1 and SHP-2, peptide competition assays were performed. The tyrosines within  $\beta c$  which become tyrosine phosphorylated upon IL-3 stimulation have not been biochemically mapped, so phosphopeptides corresponding to sequences surrounding 5 tyrosine residues within  $\beta c$  (see Table II.2) were tested for their ability to block precipitation of tyrosine phosphorylated  $\beta c$  by the SHP-1(SH2)<sub>2</sub>- and SHP-2(SH2)<sub>2</sub>- GST fusion proteins. Phosphopeptides representing tyrosines 806 and 856 were not tested as  $\beta c$  truncation mutants up to residue 763 retain normal functions in response to IL-3 such as: growth, tyrosine

phosphorylation of  $\beta c$  and Shc, Ras activation and MAP kinase activation (Sakamaki *et al.*, 1992; Quelle *et al.*, 1994). The GST fusion proteins (15  $\mu$ g SHP-1-(SH2)<sub>2</sub>-GST and 5  $\mu$ g SHP-2-(SH2)<sub>2</sub>-GST) were first preincubated with the phosphopeptides before being added to the TF-1 cell extracts from control (C) and IL-3 (3) stimulated cells. Precipitates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The results of these competition analyses are shown in Figure III.4. The phosphopeptide corresponding to the residues surrounding tyrosine 612 (pY612) inhibited the precipitation of tyrosine phosphorylated  $\beta c$  by SHP-1(SH2)<sub>2</sub>-GST (Fig. III.4 A). Some inhibition was also consistently observed with phosphopeptide pY750, but not consistently with the other phosphopeptides. Only phosphopeptide pY612 consistently inhibited the precipitation of tyrosine phosphorylated  $\beta c$  by SHP-2(SH2)<sub>2</sub>-GST (Fig. III.4 B). Reprobing this same blot with polyclonal anti- $\beta c$  antibodies confirmed that the presence of phosphopeptide pY612 had inhibited precipitation of  $\beta c$  by SHP-2(SH2)<sub>2</sub>-GST (Fig. III.4 B, lower panel). Competition required tyrosine phosphorylation of the peptide as unphosphorylated peptide did not inhibit precipitation of  $\beta c$  by either SHP-1(SH2)<sub>2</sub>- or SHP-2(SH2)<sub>2</sub>- GST fusion proteins (results not shown).

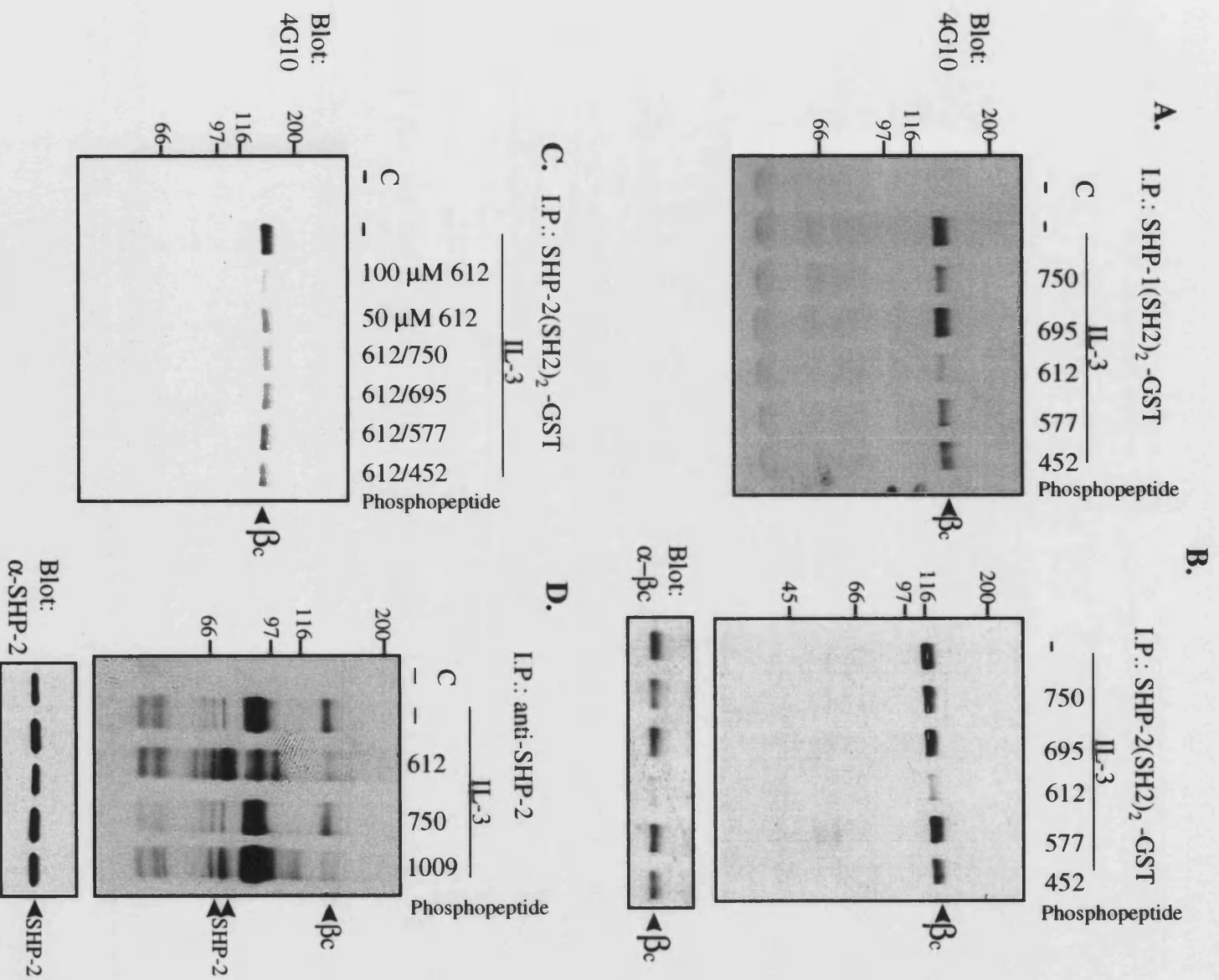
SHP-1 and SHP-2 each contain two SH2 domain which appear to differ in their functions (Pei *et al.*, 1996). Therefore, combinations of phosphopeptide pY612 with other phosphopeptides were tested to examine the possibility that the latter might make a secondary contribution to the binding of SHP-2 to  $\beta c$ . As seen in Figure III.4 C, pY612 almost completely inhibited precipitation of tyrosine phosphorylated  $\beta c$  by the SHP-2(SH2)<sub>2</sub>-GST fusion protein at a concentration of 100  $\mu$ M. At 50  $\mu$ M phosphopeptide pY612, inhibition was approximately 80%. When 50  $\mu$ M pY612 and 50  $\mu$ M of the other phosphopeptides were combined (Fig. III.4 C), the reduction in the amount of tyrosine phosphorylated  $\beta c$  precipitated by SHP-2(SH2)<sub>2</sub>-GST was similar to that observed in the presence of 50  $\mu$ M pY612 alone. Therefore, it appears that the SH2 domains of SHP-2 interact solely with residues surrounding tyrosine 612 of  $\beta c$ . Technical difficulties have prevented clear-cut results from being obtained in similar experiments with SHP-1.

#### FIGURE III.4

##### **Phosphopeptide pY612 inhibits binding of SHP-1 and SHP-2 to $\beta c$ .**

TF-1 cells were either left untreated as a control (C) or stimulated for 10 minutes with IL-3. (A, B), cell extracts from the equivalent of  $1 \times 10^7$  cells per sample were incubated in the absence (-) or the presence of 100  $\mu\text{M}$  of the indicated phosphopeptides and SHP-1(SH2)<sub>2</sub>-GST (A) and SHP-2(SH2)<sub>2</sub>-GST (B) precipitates prepared. "Pre" indicates samples removed prior to precipitation. The results in A and B are representative of three and four individual experiments respectively. (C), cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100  $\mu\text{M}$  and 50  $\mu\text{M}$  phosphopeptide pY612 or a combination of 50  $\mu\text{M}$  phosphopeptide pY612 and 50  $\mu\text{M}$  pY750, pY695, pY577, or pY452, and SHP-2(SH2)<sub>2</sub>-GST precipitates prepared. These results are representative of 3 experiments. (D), cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 500  $\mu\text{M}$  of the indicated phosphopeptides and anti-SHP-2 immunoprecipitates prepared. These results are representative of two separate experiments. All samples were separated on 7.5% acrylamide gels by SDS-PAGE and immunoblotting was performed with 4G10 anti-phosphotyrosine antibodies. The same blot as in B was stripped and reprobed with polyclonal anti- $\beta c$  antibodies (lower panel). The same blot as in D was stripped and reprobed with polyclonal anti-SHP-2 antibodies (lower panel). Molecular mass standards are shown in kDa and the positions of  $\beta c$  and SHP-2 are indicated.

**FIGURE III.4**



Phosphopeptide competition analyses of anti-SHP-2 immunoprecipitates from control and IL-3-treated TF-1 cells were also performed to investigate whether endogenous SHP-2 could also be inhibited from binding to tyrosine phosphorylated  $\beta c$  by phosphopeptide pY612. A phosphopeptide incorporating the residues surrounding tyrosine 1009 within the PDGFR (DTSSVLpYTAVQPN; Dechert *et al.*, 1995a) has previously been shown to be the binding site for SHP-2 (Lechleider *et al.*, 1993b; Kazlauskas *et al.*, 1993) and was used as a control. Cells were lysed in the presence of 500  $\mu$ M phosphopeptide pY612 or pY750 or 100  $\mu$ M pY1009 and precipitations prepared with the anti-SHP-2 antibody. Phosphopeptides pY612 and pY1009 significantly reduced the co-precipitation of endogenous SHP-2 with tyrosine phosphorylated  $\beta c$  from IL-3 stimulated cells, whereas pY750 did not (Fig. III.4 D). Figure III.4 D lower panel shows the blot to be evenly loaded with respect to SHP-2. Higher concentrations of phosphopeptide were required to inhibit co-precipitation of  $\beta c$  by anti-SHP-2 antibodies compared with the fusion proteins, perhaps reflecting a high affinity complex between SHP-2 and  $\beta c$ . Competition experiments were attempted using the anti-SHP-1 antibody, but proved technically challenging. The anti-SHP-1 antibody appears to be much less efficient in immunoprecipitating and the amount of tyrosine phosphorylated  $\beta c$  Co-precipitated was often low. This could be explained if the association between SHP-1 and the receptor is of low affinity or transient in nature.

Localisation of the PTPases SHP-1 and SHP-2 to  $\beta c$  in response to IL-3 may provide a means of positively or negatively regulating cell growth and differentiation resulting from the dephosphorylation of key signalling molecules located at or near the membrane. As SHP-2 has previously been observed to be associated with Grb2 and the p85 subunit of PI3-K after IL-3 stimulation (Welham *et al.*, 1994b), the PTPases may also function as adaptor proteins and perform multiple functions in IL-3 signalling pathways.

### **III.E. Discussion.**

The data presented here demonstrate that the tyrosine phosphatases SHP-1 and SHP-2 can both inducibly bind to  $\beta c$  following IL-3 stimulation. This association appears to be directly mediated by interactions between the SH2 domains of SHP-1 and SHP-2 and phosphotyrosine residues within  $\beta c$ . A phosphotyrosine-containing peptide based on sequences surrounding tyrosine 612 of  $\beta c$  was able to compete the binding of both SHP-1(SH2)<sub>2</sub>-GST and SHP-2(SH2)<sub>2</sub>-GST fusion proteins to tyrosine phosphorylated  $\beta c$  in *in vitro* assays and also the binding of endogenous SHP-2 to  $\beta c$  in immunoprecipitation studies. These results strongly suggest that the SH2 domains of both SHP-1 and SHP-2 bind to residues surrounding tyrosine 612 of  $\beta c$ .

Tyrosine 612 of  $\beta c$ , to which the SH2 domains of SHP-1 and SHP-2 have been demonstrated in this study to bind, is located within the motif LEYLCLP, which has similarities to motifs previously identified for SHP-1 and SHP-2 SH2 interactions. The amino-terminal SH2 domain of SHP-1 showed a broad selectivity for pY-hydrophobic-X-hydrophobic motifs from a phosphopeptide library (Songyang *et al.*, 1994). In addition, tyrosine 429 of the EpoR, which lies in the pYLYL motif, has been shown to be essential for the SHP-1 binding (Klingmuller *et al.*, 1995a). Pei *et al.* (1994) have previously shown that a phosphopeptide based on the sequence surrounding tyrosine 612 of  $\beta c$  (referred to as tyrosine 628, which includes the 14-amino acid signal peptide) bound the amino-terminal SH2 domain of SHP-1, activated the phosphatase, and acted as a substrate for SHP-1. They suggested this tyrosine may be the binding site for SHP-1 to  $\beta c$  (Pei *et al.*, 1994), and the results presented here show a similar peptide does compete with SHP-1 for binding to  $\beta c$ . A weaker inhibition of precipitation of  $\beta c$  with SHP-1(SH2)<sub>2</sub>-GST by phosphopeptide 750 was observed. Tyrosine 750 is located in the sequence pYVEL, which also conforms to the predicted SHP-1 SH2 binding motif.

The selectivity of the amino-terminal SH2 domain of SHP-2, determined using a degenerate peptide library, was shown to be pY(V/I/T)X(V/L/I) (Case *et al.*, 1994). Experiments using both mutant receptors (Kazlauskas *et al.*, 1993) and peptide competition

assays (Lechleider *et al.*, 1993b) demonstrate that tyrosine 1009 of the PDGFR, in the motif pYTAV, is required for SHP-2 binding. In addition, using EpoR mutant receptors, it has been shown that SHP-2 binds, via its SH2 domains, to the activated EpoR at tyrosine 425, in the motif pYTIL (Tauchi *et al.*, 1996). The residues surrounding tyrosine 612 (YLCL) of  $\beta c$  are similar to these previously described binding motifs for SHP-2.

The effects of mutagenesis of tyrosines 612 and 750 of  $\beta c$  on tyrosine phosphorylation of substrates in response to GM-CSF have been reported (Inhorn *et al.*, 1995; Durstin *et al.*, 1996). In these transfectants, normal levels of SHP-2 tyrosine phosphorylation were observed (Durstin *et al.*, 1996). However, the association of SHP-2 with  $\beta c$  was not examined in these mutant  $\beta c$ -expressing cells. An interesting point relating to this is whether stable association of SHP-2 with  $\beta c$  is required for its tyrosine phosphorylation. The data presented here suggest that in the presence of phosphopeptide pY612 and pY1009, which compete for the binding of SHP-2 to  $\beta c$ , the levels of SHP-2 tyrosine phosphorylation are not diminished and actually appear to increase (Fig III.4 D), supporting the notion that SHP-2 does not need to be bound to  $\beta c$  to become phosphorylated. In fact, it appears that PDGF-stimulated tyrosine phosphorylation of SHP-2 does not require SHP-2 to stably associate with the receptor as PDGF receptor mutants that associate poorly with SHP-2 were able to mediate tyrosine phosphorylation of SHP-2 (Kazlauskas *et al.*, 1993). The increased SHP-2 tyrosine phosphorylation observed may be caused by activation of a kinase involved in phosphorylation of SHP-2. However, one can not rule out the possibility that SHP-2 associates transiently with  $\beta c$  and that this is all that is required for its phosphorylation. The apparent decrease in tyrosine phosphorylation of the p90 protein and the appearance of an additional tyrosine phosphorylated protein of 97 kDa in the anti-SHP-2 immunoprecipitations incubated with phosphopeptide 612 (Figure III.4 D) are difficult to explain at present. Perhaps a conformational change in SHP-2 induced by the peptide, reduced SHP-2's affinity for the tyrosine phosphorylated p90 protein and increased its affinity for the 97 kDa protein. Additional mutational analyses of  $\beta c$  have implicated two regions of  $\beta c$  which appear to influence SHP-2 tyrosine phosphorylation (Itoh *et al.*, 1996). Mutation of tyrosine 577 in conjunction with a truncation up to residue 589 resulted in greatly reduced levels of SHP-2

tyrosine phosphorylation in response to GM-CSF, although either mutation alone had no effect (Itoh *et al.*, 1996). The results presented here suggest that tyrosine 577 is not involved in SHP-2 binding to  $\beta c$  and tyrosine 612 is the major site of interaction. Since tyrosine 612 is removed in the 589  $\beta c$  truncation mutant, but SHP-2 is still tyrosine phosphorylated, it may be that association of SHP-2 is not required for its tyrosine phosphorylation, and the data presented here are consistent with this possibility.

As a part of this study, the same synthetic phosphopeptides used in the competition experiments were tested by Dr. Ute Dechert (formerly of Biomedical Research Centre, Vancouver, B.C., Canada) in phosphatase assays to investigate whether they could also serve as substrates for SHP-1 and SHP2 (Bone *et al.*, 1997). Interestingly, phosphopeptide pY612, which competed the binding of both SHP-1 and SHP-2 to  $\beta c$  was also determined to be the best substrate for SHP-1 and SHP-2 catalytic activities (Ute Dechert; Bone *et al.*, 1997). It has been shown previously that SHP-1 and SHP-2 prefer substrates that have acidic residues to the amino-terminus of the phosphotyrosine (Dechert *et al.*, 1995; Sugimoto *et al.*, 1993), and this is the case for the residues surrounding tyrosine 612. Whether this site is an *in vivo* substrate remains to be determined, although Yi *et al.*, (1993) reported the SHP-1-catalysed dephosphorylation of Aic2A. Therefore, the same site that appears to be recognised by the SH2 domains of these phosphatases also appears to be utilised as a substrate. In experiments using full-length recombinant SHP-2 and peptides for substrates, Sugimoto *et al.* (1993) found SHP-2 to have preference for tyrosine 1009 (to which the SH2 domain of SHP-2 binds, leading to its activation (Lechleider *et al.*, 1993b)) and tyrosine 1021 of the PDGFR, suggesting that tyrosine 1009 may both regulate and act as a substrate for the PTPase activity of SHP-2. Similar substrate specificities were observed with PDGF receptor peptides (Dechert *et al.*, 1995), and the results with SHP-2 and  $\beta c$  suggest a similar mechanism may be used in IL-3 signalling. However, using immunoprecipitated phosphorylated PDGF $\beta$  receptor and recombinant full-length SHP-2, phosphotyrosines 771 and 751, followed by tyrosine 740, were dephosphorylated preferentially, while tyrosine 1021 and tyrosine 1009 were reported to be very poor substrates (Klinghoffer *et al.*, 1995). These discrepancies could arise from differences in using the intact PDGF  $\beta$  receptor, which contains multiple potential phosphorylation sites, which may bind other proteins and mask potential binding sites, instead



of using peptides as substrates. In the *in vitro* assay system used to determine the best phosphopeptide substrate for SHP-1 and SHP-2 as a part of the study presented here, the SHP-1 and SHP-2 recombinant enzymes used lacked their SH2 domains, thus removing any potential “activating” effects of the various phosphopeptides (Ute Dechert; Bone *et al.*, 1997).

As demonstrated in this study, both SHP-1 and SHP-2 can directly associate with  $\beta c$  in an IL-3-dependent manner. However, it is likely that SHP-1 and SHP-2 have different roles when localised to the IL-3 receptor. SHP-1 is thought to be a negative regulator of growth and functions to terminate signals. It appears to negatively regulate signals in different ways. SHP-1 has been shown to directly dephosphorylate receptors following activation. Overexpression of SHP-1 in DA3 cells has lead to a decrease in Aic2A tyrosine phosphorylation (Yi *et al.*, 1993). Additionally, in studies on macrophages from *me/me* mice, which are hyper-responsive to CSF-1, the CSF-1R becomes hyper-phosphorylated upon CSF-1 stimulation, suggesting that SHP-1 dephosphorylates the CSF-1R (Chen *et al.*, 1996). Alternatively, SHP-1 has also been shown to inactivate the receptor-associated tyrosine kinases. The binding of SHP-1 to the EpoR has been shown to activate the PTPase, leading to dephosphorylation Jak-2, and resulting in the termination of proliferative signals (Klingmuller *et al.*, 1995). SHP-1 has also been shown to interact directly with Jak-2, leading to its dephosphorylation (Jiao *et al.*, 1996). IL-3 also induces activation of Jak-2 (Silvennoinen *et al.*, 1993), so SHP-1 may function in a similar manner in IL-3-signal transduction.

Also competing for the same binding site on  $\beta c$  is SHP-2, which is thought to act as a positive mediator of growth factor signals. SHP-2 has been hypothesised to play a positive role in signal transduction by serving as an adaptor protein between the receptor and Grb2. The SH2 domain of Grb2 is predicted to bind to a consensus sequence pYXNX (Songyang *et al.*, 1994) of which there are two in SHP-2: tyrosine 304 (pYINA) and tyrosine 542 (pYTNI). Tyrosine 542 has been reported to be the major *in vivo* site of tyrosine phosphorylation on SHP-2 in response to PDGF (Bennett *et al.*, 1994). SHP-2 has been shown to associate with Jak-1 and Jak-2, which results in phosphorylation of tyrosine 304 of SHP-2, leading to the creation of a Grb2 SH2 domain recognition motif (Yin *et al.*, 1997). It has been shown previously that tyrosine-phosphorylated SHP-2 associates with the adaptor molecule Grb2 following treatment of cells with PDGF (Li *et al.*, 1994; Bennett *et al.*, 1994), Epo (Tauchi *et*

*al.*, 1994 p25206), and SLF (Tauchi *et al.*, 1995) and can itself also bind to the respective receptors (PDGFR, EpoR and *c-kit*). In response to IL-3, tyrosine phosphorylated SHP-2 has been shown to associate with the SH2 domain of Grb2 and phosphopeptides based on sequences surrounding tyrosine 304 and 542 were shown to almost completely inhibit binding of a Grb2SH2-GST fusion protein to SHP-2 (Welham *et al.*, 1994b). The results presented here suggest that SHP-2 can associate directly with  $\beta c$ . Thus SHP-2 may act as an adaptor in IL-3 signalling by associating with  $\beta c$  and Grb2, thereby leading to activation of the Ras/MAP kinase pathway, known to be activated by IL-3 (Duronio *et al.*, 1992b; Welham *et al.*, 1994b) (see Fig. III.5).

Additionally, it has been shown previously, in murine cells, that the p85 subunit of PI3-K can be co-precipitated with SHP-2 after activation of cells with IL-3 (Welham *et al.*, 1994b). The interaction between SHP-2 and p85(PI3-K) appeared complex and was suggested not to be mediated directly by the SH2 domains of the p85 subunit. A major tyrosine phosphorylated 100 kDa protein that directly interacted with SHP-2 in IL-3-stimulated murine cells was also shown to directly interact with p85(PI3-K) (Craddock and Welham, 1997). Thus, SHP-2 may be involved in activation of the PI3-K pathway by recruiting a p100-p85(PI3-K) complex to the IL-3 receptor and hence facilitate translocation of PI3-K to the vicinity of its lipid substrates (see Fig. III.5). The 100 kDa protein was also found to be a substrate for SHP-2 (Craddock and Welham, 1997). Therefore, SHP-2 may also function to regulate these interactions. Thus, modulation of SHP-2 activity and adaptor function could also affect PI3-K activation.

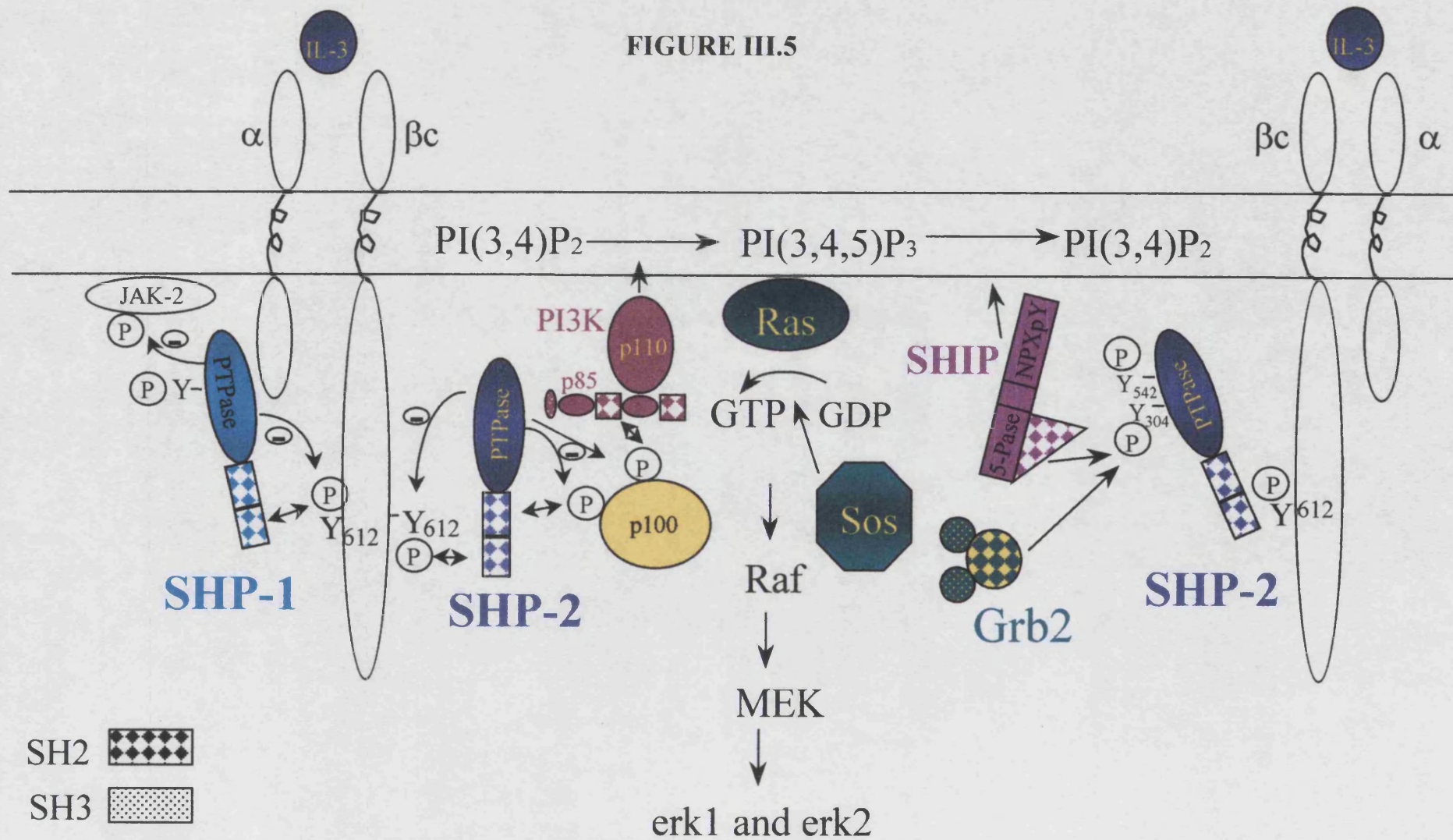
Recently, this 100 kDa protein has been cloned from Ba/F3 cells and has been termed Gab2 (Gu *et al.*, 1998). It has been shown that Gab2 associates with SHP-2 and the p85 subunit of PI3-K, consistent with the results presented here. Gab2 is a scaffolding molecule distantly related to *Drosophila* Daughter of Sevenless (DOS), a substrate for the *Drosophila* SHP-2 homologue Corkscrew (CSW) which acts downstream of the receptor tyrosine kinase Sevenless and upstream of or in parallel to the Ras pathway, and mammalian Gab1 (Gu *et al.*, 1998). Gab1 was originally isolated as a binding protein for Grb2 and is tyrosine phosphorylated and interacts with SHP-2 and PI3-K in response to various growth factor stimuli. Gab2, like Gab1 and DOS, contains an amino-terminal PH domain, proline-rich

sequences, and multiple tyrosine residues (Gu *et al.*, 1998). Expression of Gab2 mutants unable to bind SHP-2 blocks cytokine-induced *c-fos* promoter activation but does not inhibit MAP kinase activation. However, expression of dominant negative SHP-2 inhibits IL-3-induced MAP kinase activation and *c-fos* activation in Ba/F3 cells in response to IL-3 (Gu *et al.*, 1998). Thus, SHP-2 appears to act at two or more sites in IL-3 signalling: (1) the requirement for SHP-2 for MAP kinase activation is mediated through a Gab2-independent mechanism, whereas (2) the association of SHP-2 with Gab2 is a requirement for transcriptional activation (Gu *et al.*, 1998).

SHP-2 has also been shown to bind the inositol polyphosphate 5-phosphatase SHIP whose catalytic activity can act to dephosphorylate the primary PI3-K product, PI(3,4,5)P<sub>3</sub>, to PI(3,4)P<sub>2</sub> (Damen *et al.*, 1996; Liu *et al.*, 1997b). The association of SHP-2 with SHIP has been shown to occur through the direct association of the SH2 domain of SHIP with a pYXN(I/V) sequence within SHP-2 which also serves as a Grb2 binding site (Welham *et al.*, 1994b; Liu *et al.*, 1997b). Thus, SHP-2 could also function in localising SHIP to the IL-3 receptor, in the vicinity of its lipid substrates (see Fig. III.5). SHIP has also been suggested to play a negative role in growth factor mediated signalling (Lioubin *et al.*, 1996; Chacko *et al.*, 1996; Liu *et al.*, 1997a). One could speculate that SHIP could act as a negative regulator by competing with Grb2 for binding to SHP-2. This could result in localisation of SHIP to the vicinity of its substrates, possibly leading to down-regulation of PI3-K-induced proliferation and survival signals. Since SHP-2 acts as a positive regulator, the binding of SHP-2 to SHIP may serve to dephosphorylate and inactivate SHIP.

In summary, the results presented here demonstrate that in response to IL-3, SHP-1 and SHP-2 bind through their SH2 domains to tyrosine 612 of  $\beta c$ . The regulation of signalling pathways by SHP-1 and SHP-2 is complex and a proposed model is outlined in Figure III.5. The association of SHP-2 with  $\beta c$  may function in localising signalling molecules to the plasma membrane. The recruitment of Grb2 by SHP-2 may lead to activation of the Ras/MAP kinase pathway whereas localisation of a p100-p85(PI3-K) complex may function in activating pathways mediated by PI3-K. Alternatively, SHP-2 may recruit SHIP to the receptor, possibly leading to the down-regulation of PI3-K activated pathways, due to the catalytic activity of SHIP, or lead to the down-regulation of the Ras/MAP kinase pathway by competing with Grb2

FIGURE III.5



Model: the role of SHP-1 and SHP-2 in mediating IL-3 signalling pathways

for binding to SHP-2. Binding of SHP-1 to  $\beta c$  may lead to the dephosphorylation and inactivation of Jak-2 kinase, resulting in the termination of proliferative signals. Although both SHP-1 and SHP-2 associate with tyrosine 612 of  $\beta c$ , it is likely that only a portion of the receptors are associated with SHP-1 and SHP-2 at any one time. In addition, both SHP-1 and SHP-2 appear to be able to regulate their own binding to the receptor as phosphopeptide pY612 also served as a substrate for the catalytic domain of both the PTPases. Therefore, the activation/deactivation of each phosphatase likely leads to a complex modulation of signalling pathways regulated by phosphorylation/dephosphorylation events and any shifting of the equilibrium between the two phosphatases would result in either a positive or negative effect on IL-3-induced signals.

## **CHAPTER IV**

### **Protein-Protein Interactions Mediated by Shc in response to IL-3**

#### **IV.A Introduction and Aims.**

p52<sup>Shc</sup> and p46<sup>Shc</sup> are two of the major substrates for IL-3-induced protein tyrosine kinases and it therefore seems likely that Shc would be involved in controlling signalling pathways stimulated in response to IL-3. Tyrosine phosphorylation of Shc at position 317 creates a binding site for the SH2 domain of Grb2, linking Shc with the activation of the Ras/MAP kinase pathway. Shc has been shown to associate with the tyrosine phosphorylated EGFR via its PTB (Blaikie *et al.*, 1994) and SH2 (Pelicci *et al.*, 1992) domains following EGF stimulation, resulting in significant relocalisation of Shc-Grb2-Sos complexes to the plasma membrane and hence to the vicinity of Ras (Ruff-Jamison *et al.*, 1993). However, no mass translocation of Shc to the plasma membrane after IL-3 stimulation has been observed, although, a slight increase in tyrosine phosphorylated Shc at the membrane has been detected (Welham *et al.*, 1994a). Therefore, Shc may have functions other than as an adaptor protein involved in localising Grb2 to the membrane in response to IL-3 and may play important roles in other signalling pathways.

Shc, via its PTB domain, has also been shown to associate with a tyrosine phosphorylated 145 kDa protein, now known to be the inositol phosphatase SHIP (Lioubin *et al.*, 1996; Damen *et al.*, 1996). SHIP has been shown to selectively hydrolyse the 5'-phosphate from inositol 1,3,4,5-tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) (Damen *et al.*, 1996; Lioubin *et al.*, 1996). Thus, Shc may also play a role in pathways involved with regulation of lipid metabolism.

One way to investigate the roles of Shc in IL-3 signal transduction is to examine the protein-protein interactions mediated by the various domains of Shc and this was the primary aim of this part of the study. This may provide information into the possible mechanisms of Shc activation of the Ras/MAP kinase pathway, Shc regulation of lipid metabolism or other functions of Shc in response to IL-3.

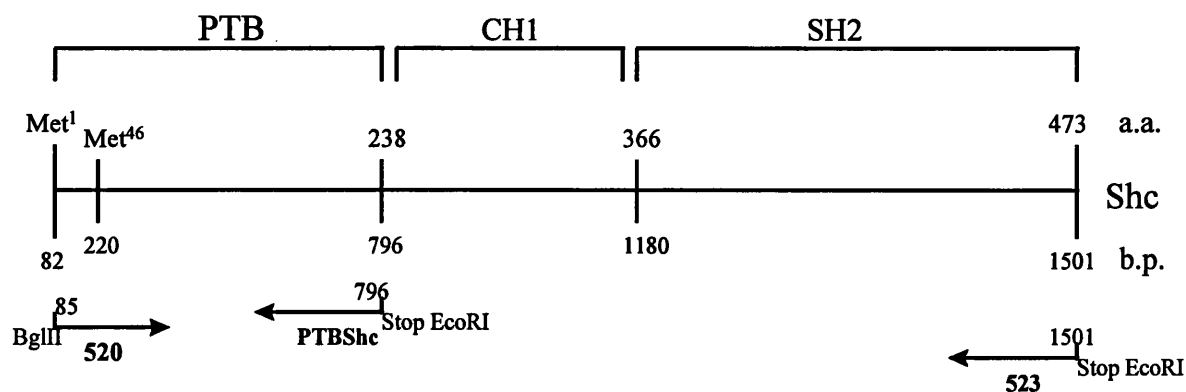
#### **IV.B Preparation of Shc-GST fusion proteins.**

Using a human p52<sup>Shc</sup> cDNA containing plasmid (plasmid B15: human Shc cDNA in pGEM3 vector, a gift of Dr. Tony Pawson, Toronto, Canada) as a template, DNA fragments corresponding to various Shc domains were synthesised by polymerase chain reaction (PCR) (section II.A.13). The oligonucleotide primers (Pharmacia Biotech) used in the synthesis of FL Shc and the individual Shc PTB domain are outlined in Figure IV.1 BglII and EcoRI sites were engineered into the oligonucleotide primer for ease of cloning the amplified Shc fragments into pGEX2T. The amplified DNA was isolated, digested with BglII and EcoRI and cloned into BamHI and EcoRI restricted pGEX2T (Pharmacia Biotech). The SH2 domain (amino acids 366-473) of Shc cloned into pGEX2T (pGSTShcSH2) was a gift of Dr. Tony Pawson (Toronto, Canada). Ligations were transformed into XL-1BL (section II.A.4) and recombinants screened for the presence of the correct insert by restriction enzyme mapping. Large-scale cultures of XL-1BL containing the vectors with the correct inserts were grown, induced overnight with isopropyl  $\beta$ -thiogalactopyranoside (IPTG) and the GST fusion proteins purified on glutathione Sepharose beads (see section II.C.3). Figure IV.2 depicts the various Shc GST fusion proteins constructed and used for the following experiments.



**FIGURE IV.1**

**Oligonucleotides used for the cloning of various domains of Shc**



**520:** 5' sense oligonucleotide with Bgl II site for cloning, beginning at bp 85 of Shc.

/85  
5' TAG AGA TCT AAC AAG CTG AGT GGA  
Bgl II

**PTBShc:** 3' antisense oligonucleotide with Stop site and EcoRI site for cloning.

Ends at bp 796 of Shc

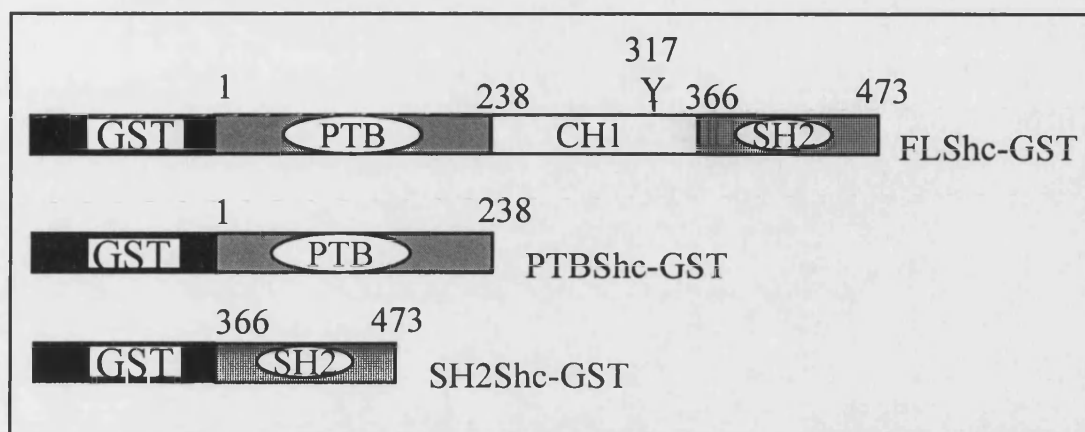
/796  
3' GGA CTG GTA GTC ACT CTT AAG T  
Gln Stop EcoRI

**523:** 3' antisense oligonucleotide with Stop and EcoRI site for cloning. Ends at bp 1501 of Shc

/1501  
3' CAC CTC GCC TTT GAC ACT CTT AAG GAT  
Leu Stop EcoRI

**FIGURE IV.2**

**Schematic representation of p52<sup>Shc</sup> domains expressed as GST-fusion proteins.**

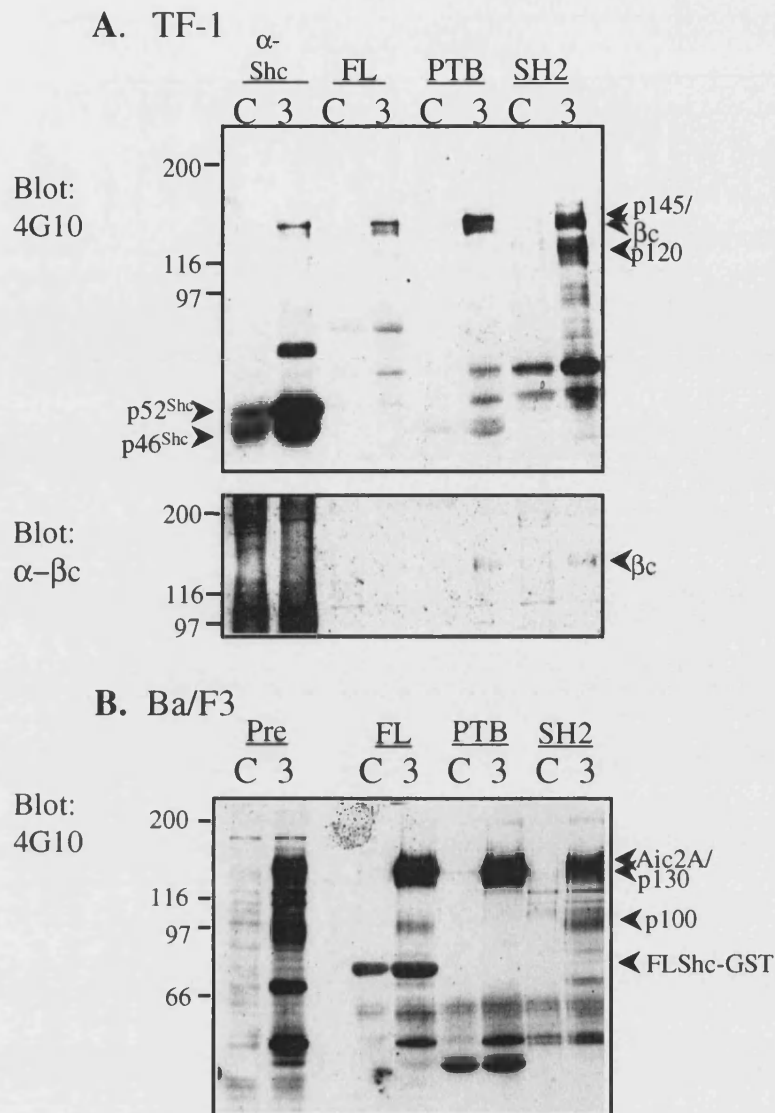


#### **IV.C Precipitation of tyrosine phosphorylated proteins from IL-3 stimulated cells by Shc-GST fusion proteins.**

Using GST fusion proteins of the various domains of Shc, interactions between Shc and other phosphoproteins were investigated. The various GST fusion proteins (Fig. IV.2) were used for direct precipitation analyses from TF-1 and Ba/F3 cells that had either been left untreated as a control (C) or stimulated with IL-3 for 10 minutes (3). The precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the 4G10 anti-phosphotyrosine monoclonal antibody. The results are shown in Figure IV.3.

In TF-1 cells stimulated with IL-3, the anti-Shc antibody precipitated what appeared to be two distinct tyrosine phosphorylated proteins: a protein appearing as a broad 135-140 kDa band and a sharper, higher molecular weight 145 kDa protein (Fig IV.3 A). The full length (FL) Shc-GST fusion protein precipitated a similar pattern of proteins from IL-3 stimulated cell extracts as did the PTB domain alone. However, only the single broad 135-140 kDa tyrosine phosphorylated band was precipitated by the SH2Shc-GST fusion protein in addition to a broad 120 kDa protein of unknown identity. Reprobing this same blot with anti- $\beta$ c antibodies confirmed that one of the proteins precipitated by the FLShc-, PTBShc- and

**FIGURE IV.3**



**Shc-GST precipitations from TF-1 and Ba/F3 cells.**

(A) TF-1 cells and (B) Ba/F3 cells were deprived of factor and serum then either left untreated as a control (C) or treated for 10 minutes with IL-3 (3). Cell extracts from the equivalent of  $1 \times 10^7$  cells/sample were precipitated with either the polyclonal anti-Shc antibody, or the FL-, PTB-, or SH2- Shc-GST fusion proteins. Bound proteins were eluted and separated by SDS-PAGE through a 7.5% acrylamide gel. Immunoblotting was performed with 4G10 anti-phosphotyrosine antibodies. The same immunoblot in A was stripped and reprobed with antibodies against  $\beta$ c (lower panel). The molecular mass standards are shown in kDa and the positions of the 130-145 kDa proteins,  $\beta$ c, p100 and FLShc-GST are indicated by the arrows. These data are representative of 3 individual experiments.

SH2Shc- GST fusion proteins was the human IL-3 receptor  $\beta$  subunit,  $\beta$ c (Fig. IV.3 A, lower panel). It is difficult to discern  $\beta$ c in the anti-Shc immunoprecipitates due to cross-reactivity between the rabbit precipitating and blotting antibodies. The  $\beta$ c band is also difficult to detect in FLShc-GST precipitates, most likely due to lower amounts of  $\beta$ c precipitated by the FLShc-GST fusion protein. These results are representative of three separate experiments.

In IL-3-stimulated Ba/F3 cells, a similar scenario was observed (Fig. IV.3 B). After IL-3 stimulation, the FLShc-GST fusion protein appeared to precipitate at least two distinct tyrosine phosphorylated proteins: a broad 135-145 kDa band and a sharper, slightly lower molecular weight 130 kDa protein (see Figure IV.5 B for a shorter exposure of a similar experiment highlighting the different protein species precipitated in this region). Again, the Shc PTB domain alone precipitated both these proteins from IL-3-stimulated Ba/F3 extracts but the Shc SH2-GST fusion protein only precipitated the broad 135-145 kDa band. This broad band is most likely Aic2A, the mouse IL-3 receptor  $\beta$  subunit. Aic2A is a glycoprotein of 135 kDa and appearance of a broad band on an acrylamide gel is characteristic of a highly glycosylated protein. These data are representative of four individual experiments. Unfortunately, the two anti-Aic2A antibodies that were available to us were not sensitive enough to confirm the identity of this protein and were not suitable for blotting. In addition, both the FLShc-GST and SH2Shc-GST fusion proteins, but not the PTBShc-GST fusion protein, precipitated a tyrosine phosphorylated 100 kDa protein from IL-3-stimulated cell extracts.

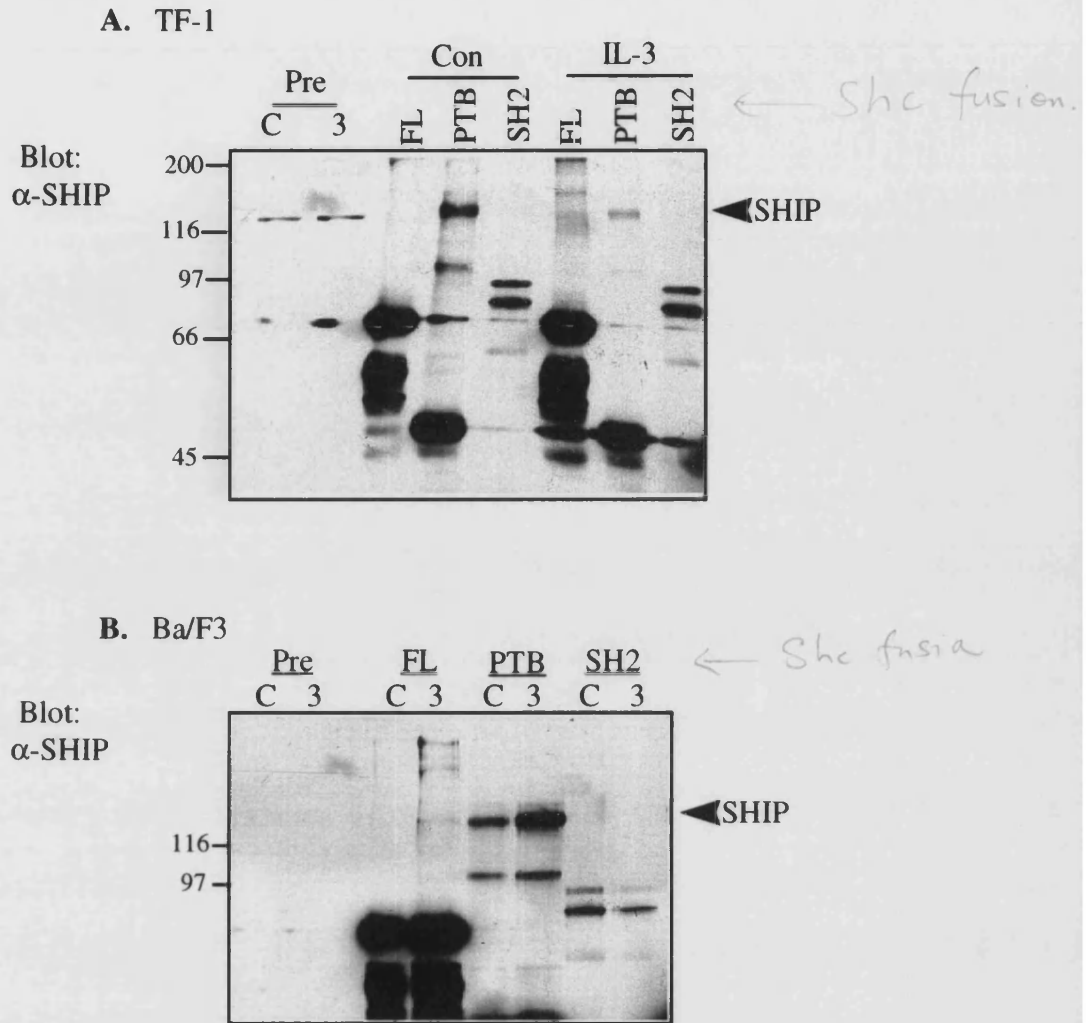
Collectively, these results suggest that after IL-3 stimulation, FL Shc can associate with tyrosine phosphorylated  $\beta$ c and a 145 kDa protein in a human cell line and a broad 135-145 kDa protein, believed to be Aic2A, and a 130 kDa protein in a mouse cell line. Therefore, both the PTB and SH2 domains of Shc appear to associate *in vitro* with  $\beta$ c/Aic2A, whereas the PTB domain, but not the SH2 domain, of Shc also appears to be able to associate with the sharper 145/130 kDa band in both human and murine cells respectively. Additionally, the SH2 domain of Shc appears to interact with unknown proteins of 120 kDa in TF-1 cells and 100 kDa in Ba/F3 cells.

#### **IV.D Interaction of Shc with SHIP.**

Since the FLShc- and PTBShc- GST fusion proteins precipitated, in addition to the broad receptor-like band, a distinct sharper band of 145/130 kDa in human/murine cells, it was next investigated whether this protein was the p145<sup>SHIP</sup> protein. Initially, Shc-GST precipitations from IL-3-stimulated TF-1 and Ba/F3 cells were immunoblotted with anti-SHIP antibodies (Fig. IV.4). In TF-1 cells, the anti-SHIP antibody reacted with appropriately sized bands from whole cell lysates (Pre) and PTBShc-GST precipitates (Fig. IV.4 A). SHIP was also detected in PTBShc-GST precipitations from Ba/F3 cells (Fig. IV.4 B). In both TF-1 and Ba/F3 cells, SHIP was absent from SH2Shc-GST precipitations and very little was detected in FLShc-GST precipitations. It is interesting to note that in both TF-1 and Ba/F3 cells, SHIP was precipitated by the PTBShc-GST fusion protein from both IL-3 stimulated and control cells and in fact there appears to be more SHIP precipitated from unstimulated TF-1 cells than IL-3 stimulated. Therefore, it appears that perhaps SHIP is constitutively bound to the PTB domain of Shc but then becomes tyrosine phosphorylated after IL-3 stimulation. These results were observed in at least two separate experiments.

To further investigate the interactions of Shc with SHIP, a series of anti-SHIP immunoprecipitations were performed. However, the anti-SHIP antibody available to us only precipitated SHIP from Ba/F3 cells and not from TF-1 cells. In Ba/F3 cells stimulated with IL-3, along with p52<sup>Shc</sup> and p46<sup>Shc</sup>, anti-Shc antibodies precipitate two distinct proteins of 130 and 140 kDa (Fig. IV.5 A). To determine if SHIP was a constituent of these bands, a sequential immunoprecipitation was performed. The primary anti-Shc immunoprecipitation was boiled and denatured in the presence of SDS and 2-mercaptoethanol, effectively denaturing any associated proteins. The resulting extracts were diluted 1 in 10 (so the concentration of SDS was <0.1% (w/v)) and secondary precipitations with anti-SHIP antibodies were performed (Fig. IV.5 A). Both the 130 and 140 kDa bands were reprecipitated by the anti-SHIP antibody, suggesting that both tyrosine phosphorylated 130 and 140 kDa proteins precipitated by the anti-Shc antibody were SHIP. These results were observed in two separate experiments.

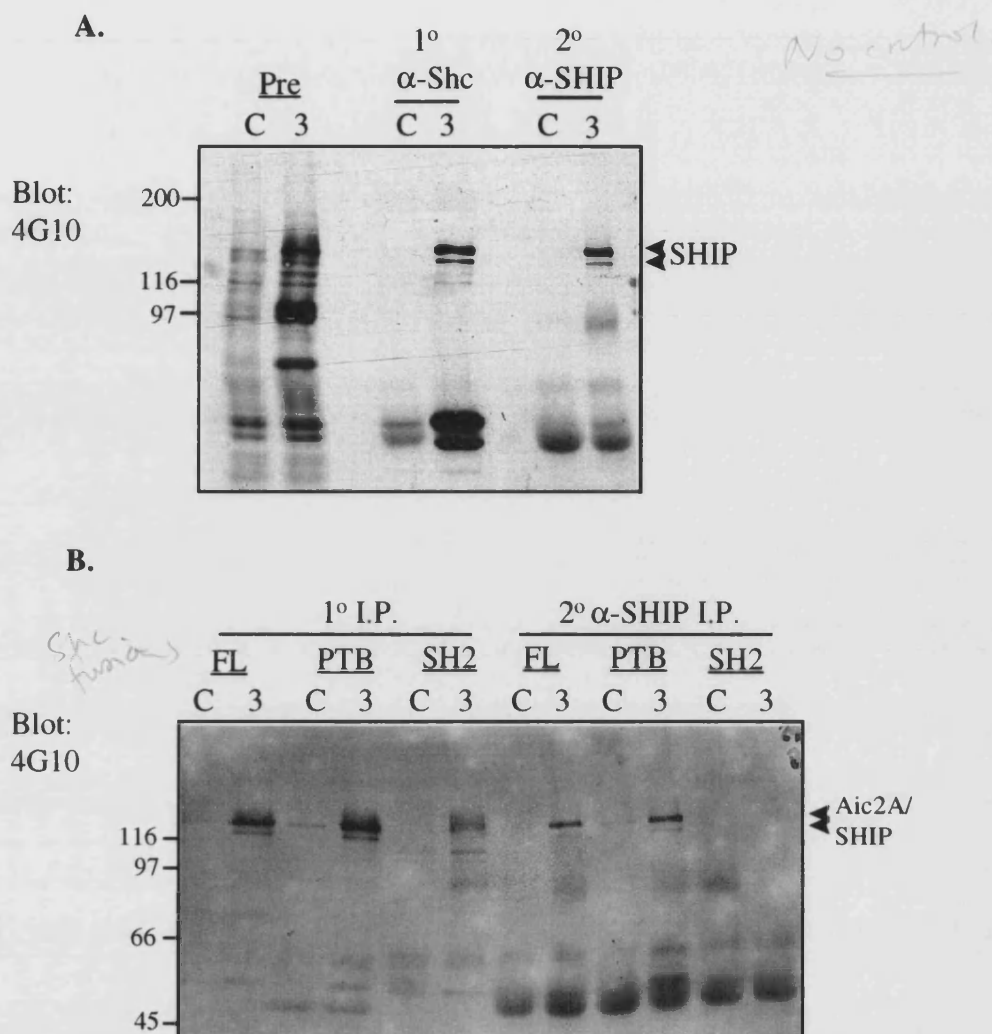
**FIGURE IV.4**



**SHIP is detected in TF-1 and Ba/F3 cells.**

Factor deprived TF-1 cells (A) or Ba/F3 cells (B) were either left untreated as a control (C) or stimulated with IL-3 for 10 minutes (3). Extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with 10  $\mu$ g of the indicated Shc-GST fusion protein. Samples were separated on a 7.5% acrylamide gel by SDS-PAGE and immunoblotted with anti-SHIP antibodies. The positions of the molecular mass standards are shown and expressed in kDa and the position of SHIP is indicated.

**FIGURE IV.5**



**SHIP interacts with the PTB domain of Shc.**

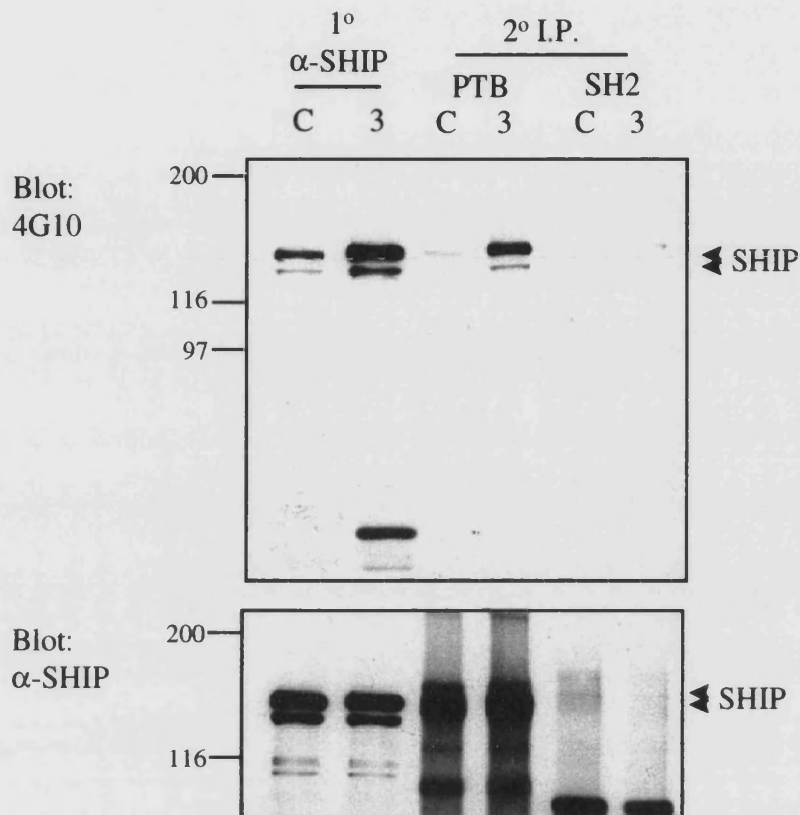
Factor deprived Ba/F3 cells were either stimulated with IL-3 for 10 minutes (3) or left untreated as a control (C). Cell extracts from the equivalent of  $2 \times 10^7$  cells/sample were precipitated with (A) 4  $\mu$ g anti-Shc antibodies or with (B) 20  $\mu$ g of either the FL-, PTB- or SH2- Shc-GST fusion proteins. Primary precipitates were eluted and denatured by boiling in SDS sample buffer and 1/10 of the sample reserved for the primary immunoprecipitation sample. After dilution, secondary precipitations were carried out using 4  $\mu$ l anti-SHIP antibody. Samples were separated on 7.5% acrylamide gels and immunoblotted with the monoclonal 4G10 anti-phosphotyrosine antibody. Molecular mass standards are shown in kDa and the position of SHIP is indicated.

In Ba/F3 cells, since the FLShc- and PTBShc- GST fusion proteins precipitated proteins in the region of 130 and 145 kDa, but one appeared to be a broad 135-145 kDa protein, thought to be Aic2A, (Fig. IV.3 B), sequential immunoprecipitations were again performed to determine if SHIP was a constituent of the tyrosine phosphorylated 130-145 kDa proteins precipitated in the Shc-GST fusion protein precipitations. The results are shown in Figure IV.5 B and were observed in two separate experiments. Primary FLShc-, PTBShc- and SH2Shc- GST fusion protein precipitations were first performed on Ba/F3 cells which had either been left untreated as a control (C) or stimulated with IL-3 for 10 minutes (3). The precipitated material was boiled and denatured. The resulting extracts were diluted and secondary precipitations with anti-SHIP antibodies were performed (Fig. IV.5 B). Two distinct sharp bands of 130 and 140 kDa were precipitated in the secondary precipitations by the anti-SHIP antibody from the initial FL and PTB Shc-GST fusion protein precipitations but not from the SH2Shc-GST precipitation. Therefore, FL Shc and the PTB domain of Shc precipitate SHIP which appears as two distinct, sharp 130 and 140 kDa bands. Thus, the broad 135-145 kDa band precipitated by FL Shc and the PTB domain of Shc is a composite of at least two proteins: the diffuse receptor band and the sharp SHIP band. The broad 135-145 kDa receptor-like band precipitated in the initial FL-, PTB, and SH2- Shc-GST precipitations was not precipitated by the anti-SHIP antibody, further evidence that this protein is Aic2A and will be referred to as thus from here on. Since these Shc-GST fusion proteins all appeared to interact with Aic2A, it seems unusual that the anti-Shc antibody only appeared to precipitate the 2 sharp SHIP band and not the broad Aic2A band (Fig. IV.5 A). This may be due to the low level of Aic2A receptors expressed on Ba/F3 cells or more likely caused by a blocking effect of the anti-Shc antibody (see section IV.E.1)

Another set of sequential immunoprecipitations were performed to show that the PTB domain of Shc can directly interact with tyrosine phosphorylated SHIP in IL-3 stimulated Ba/F3 cells. Primary anti-SHIP immunoprecipitations from control (C) and IL-3 stimulated (3) Ba/F3 cells were boiled, denatured, diluted and reprecipitated with the PTB or SH2 Shc-GST fusion protein (Fig. IV.6). These results are representative of three separate experiments. The PTB, but not the SH2, Shc GST fusion protein reprecipitated SHIP, indicating that the PTB domain of Shc can directly interact with SHIP in IL-3-stimulated Ba/F3 cells.



**FIGURE IV.6**



**The PTB domain of Shc directly interacts with SHIP.**

Factor deprived Ba/F3 cells were either left untreated as a control (C) or stimulated with IL-3 (3). Cell extracts from the equivalent of  $2 \times 10^7$  cells/sample were precipitated with 4  $\mu$ l anti-SHIP antibodies. A sample of the eluted and denatured primary precipitate was reserved for the primary immunoprecipitation sample. The remaining sample was diluted and reprecipitated with 10  $\mu$ g of either the PTB- or SH2- Shc-GST fusion protein. Samples were separated on a 7.5% acrylamide gel and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The same gel was stripped and reprobed with anti-SHIP antibodies (lower panel). The positions of SHIP and the molecular mass standards expressed in kDa are indicated.

In summary, the results described above suggest that in IL-3 stimulated TF-1 cells, one of the tyrosine phosphorylated proteins precipitated by the PTBShc-GST fusion protein appears to be SHIP, therefore suggesting that the two tyrosine phosphorylated 135-140 kDa and 145 kDa bands interacting with Shc are  $\beta c$  and SHIP respectively. In IL-3 stimulated Ba/F3 cells, SHIP appears as a sharp 130 and 140 kDa doublet which can directly interact with the PTB domain but not the SH2 domain of Shc. Multiple forms of SHIP have also been observed in B-cells (Kavanaugh *et al.*, 1996) and in murine cells (Damen *et al.*, 1998), some of which appear to be C-terminal truncations of the full length SHIP protein (Damen *et al.*, 1998). The underlying broad 135-140 kDa band precipitated by FL Shc and the individual PTB and SH2 domains in IL-3 treated Ba/F3 cells was not SHIP and is most likely Aic2A.

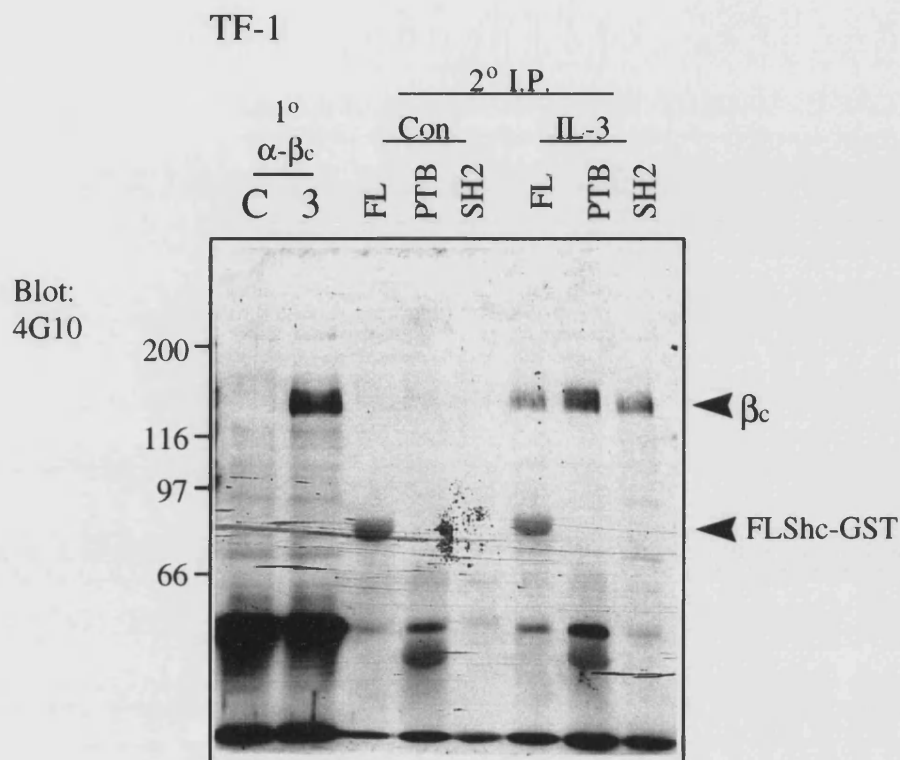
#### **IV.E Interaction of Shc with $\beta c$ /Aic2A.**

Previous reports suggest that both the SH2 and PTB domains of Shc can bind to the GM-CSFR  $\beta c$  subunit, which is shared, in humans, with the IL-3R (Lanfranccone *et al.*, 1995; Pratt *et al.*, 1996). The tyrosine residues phosphorylated on  $\beta c$  in response to GM-CSF or IL-3 have not been biochemically mapped and although both cytokines activate JAK2, it is not known if they both induce identical patterns of  $\beta c$  tyrosine phosphorylation upon receptor ligation. Therefore, the interactions mediated by the distinct domains of Shc, specifically in response to IL-3 stimulation, rather than GM-CSF, which had been used in previous studies, were examined in more detail.

##### **IV.E.1 Shc binds $\beta c$ directly through both its PTB and SH2 domains.**

Having established that in a human cell line, tyrosine phosphorylated  $\beta c$  is present in anti-Shc immunoprecipitations and Shc-GST precipitations (Fig. IV.3 A), sequential immunoprecipitation analyses were performed to determine if these interactions were direct. Extracts were prepared from TF-1 cells that had either been left untreated as a control (C) or stimulated with IL-3 (3) and immunoprecipitated with a monoclonal anti- $\beta c$  antibody. The resulting boiled and denatured primary immunoprecipitation was diluted and secondary

**FIGURE IV.7**



**Shc directly associates with tyrosine phosphorylated  $\beta_c$  through both its SH2 and PTB domains.**

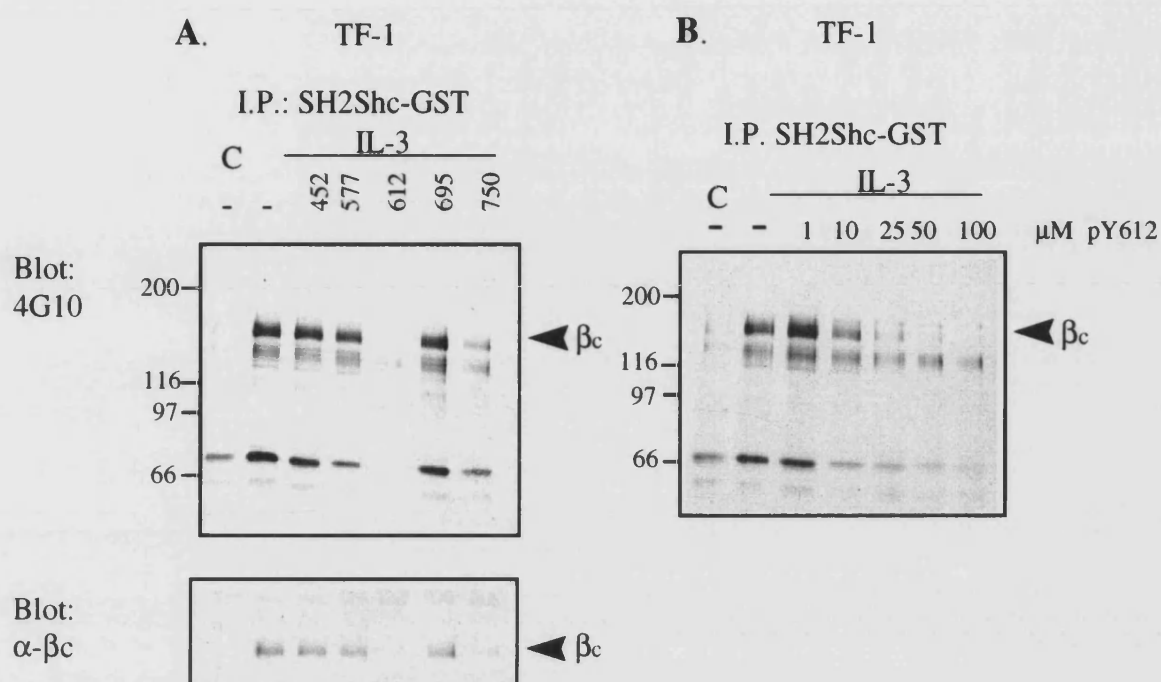
Factor deprived TF-1 cells were either left untreated as a control (C) or stimulated with IL-3 for 10 minutes (3). Cell extracts from the equivalent of  $2 \times 10^7$  cells/sample were precipitated with 20  $\mu$ g monoclonal anti- $\beta_c$  antibody. Primary anti- $\beta_c$  precipitates were eluted and denatured by boiling in SDS sample buffer and 1/10 of the sample reserved for the primary immunoprecipitation sample. After dilution, secondary precipitations were prepared with either 10  $\mu$ g of the FL-, PTB- or SH2- Shc-GST fusion proteins. The blot was probed with 4G10 anti-phosphotyrosine antibodies. Molecular mass standards are shown in kDa, and the position of  $\beta_c$  and FLShc-GST is indicated.

precipitations with FLShc-, PTBShc-, or SH2Shc- GST fusion proteins were performed. The results are shown in Figure IV.7 and were observed in two separate experiments. Tyrosine phosphorylated  $\beta$ c from cells treated with IL-3 was precipitated in the primary anti- $\beta$ c immunoprecipitation. The secondary precipitations with either FLShc-, PTBShc-, or SH2Shc GST, all resulted in reprecipitation of tyrosine phosphorylated  $\beta$ c from the IL-3 stimulated cell extracts. No tyrosine phosphorylated  $\beta$ c was precipitated from either the primary anti- $\beta$ c immunoprecipitation from unstimulated cell extracts or the secondary, unstimulated Shc-GST precipitations. The 70 kDa protein present in FLShc-GST precipitations is the FLShc-GST fusion protein itself. These results suggest that like GM-CSF (Lanfranccone *et al.*, 1995), IL-3 can induce association of Shc with tyrosine phosphorylated  $\beta$ c. This interaction appears to be mediated by both the SH2 and PTB domains of Shc. The analogous experiment in Ba/F3 cells could not be performed due to lack of an effectively precipitating anti-Aic2A antibody. However, since the SH2 domain of Shc can, by analogy, directly interact with Aic2A, this provides a reason why anti-Shc antibodies did not appear to precipitate the broad 135-145 kDa tyrosine phosphorylated Aic2A band from IL-3 stimulated Ba/F3 cells (Fig. IV.5 A) as the antibody is raised against the SH2 domain of Shc and may therefore effectively block the interaction of Shc with Aic2A.

#### **IV.E.2 Binding of the Shc SH2 domain to tyrosine phosphorylated $\beta$ c/Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 612 of $\beta$ c / tyrosine 610 of Aic2A.**

In order to provide an indication as to which of the potential tyrosines on  $\beta$ c/Aic2A was responsible for mediating the interaction with the SH2 domain of Shc following treatment with IL-3, phosphopeptide competition analyses were performed. The tyrosine residues within  $\beta$ c/Aic2A which become phosphorylated upon IL-3 stimulation have not been biochemically mapped, so phosphopeptides corresponding to the sequences surrounding 5 of the tyrosine residues within  $\beta$ c/Aic2A were tested for their ability to block precipitation of the tyrosine phosphorylated  $\beta$ c/Aic2A by SH2Shc-GST fusion protein. Table II.1 outlines the phosphopeptides used in this study. Phosphopeptides corresponding to the residues surrounding tyrosine 806 and 856 of  $\beta$ c (and the corresponding Aic2A tyrosines) again were

**Figure IV.8**



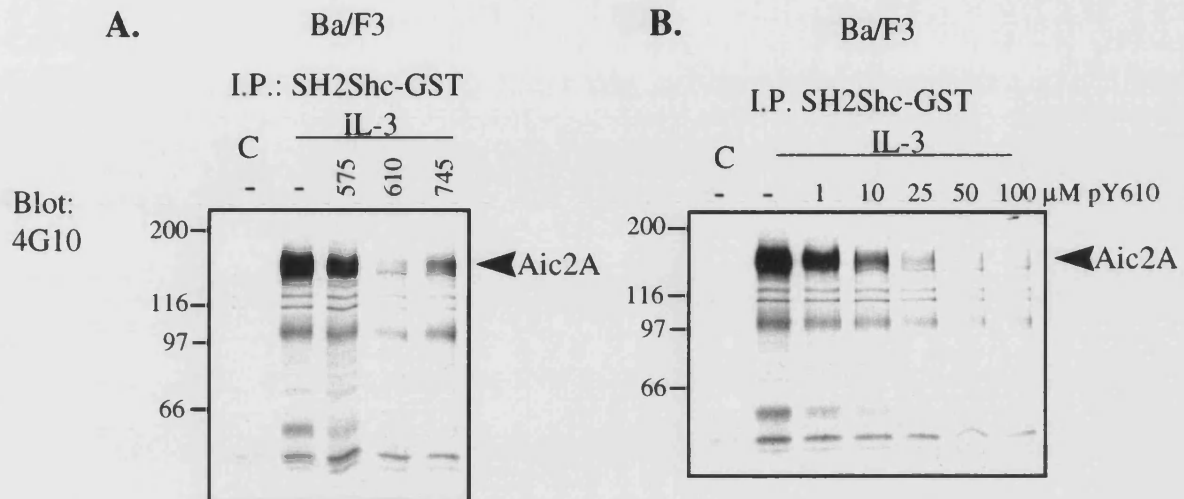
**Phosphopeptide pY612 inhibits binding of the SH2 domain of Shc to tyrosine phosphorylated  $\beta_c$ .**

Factor deprived TF-1 cells were either left untreated as a control (C) or stimulated for 10 minutes with IL-3. (A) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100  $\mu\text{M}$  of the indicated phosphopeptides and SH2Shc-GST precipitates prepared. (B) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , or 100  $\mu\text{M}$  of phosphopeptide pY612 and SH2Shc-GST precipitates prepared. The precipitates were separated by SDS-PAGE through a 7.5% acrylamide gel and immunoblotted with 4G10 anti-phosphotyrosine antibodies. Immunoblot A was stripped and reprobed with polyclonal antibodies against  $\beta_c$  (lower panel). The positions of the molecular mass standards are shown and expressed in kDa and the position of  $\beta_c$  is indicated.

not tested, as mutants of  $\beta c$  that were truncated at residue 763 or beyond retain normal functions in response to IL-3 (Sakamaki *et al.*, 1992). In TF-1 cells, only the phosphopeptide representing residues surrounding tyrosine 612 of  $\beta c$  completely inhibited the precipitation of tyrosine phosphorylated  $\beta c$  by the SH2Shc-GST fusion protein (Figure IV.8 A). The phosphopeptide representing residues surrounding tyrosine 750 also appeared to partially inhibit precipitation of  $\beta c$  (Figure IV.8 A). Reprobing this blot with polyclonal anti- $\beta c$  antibodies (Figure IV.8 A lower panel) confirmed the precipitation of tyrosine phosphorylated  $\beta c$  by the SH2Shc-GST fusion protein and the inhibition of this precipitation by phosphopeptide pY612. These results are representative of three separate experiments. Titration of phosphopeptide pY612 (Figure IV.8 B) indicated that at 25  $\mu$ M peptide the association between SH2Shc-GST and tyrosine phosphorylated  $\beta c$  was almost completely inhibited as observed in two experiments. Again, the SH2Shc-GST fusion protein also appeared to precipitate an unknown tyrosine phosphorylated 120 kDa protein whose association was also blocked by phosphopeptide pY612.

In Ba/F3 cells, the phosphopeptide representing residues surrounding tyrosine 610 of Aic2A also completely inhibited the precipitation of tyrosine phosphorylated Aic2A by the SH2Shc-GST fusion protein (Figure IV.9 A, the results are representative of three separate experiments). The phosphopeptide representing residues surrounding tyrosine 612 of  $\beta c$ , where the only difference in amino acid sequence from mouse is a Methionine instead of a Leucine in the +1 position from the phosphotyrosine, also completely inhibited precipitation of tyrosine phosphorylated Aic2A by the SH2Shc-GST fusion proteins (results not shown). Phosphopeptide pY745 also partially inhibited precipitation of Aic2A (Fig. IV.9 A). The corresponding  $\beta c$  peptide pY750 also partially inhibited the precipitation of Aic2A, while  $\beta c$  peptides pY452, 577, 695 did not (results not shown). Again, association between SH2Shc-GST and tyrosine phosphorylated Aic2A was almost completely inhibited with 25  $\mu$ M phosphopeptide pY610, as observed in two experiments (Figure IV.9 B). The interaction between the 100 kDa protein and the SH2 domain of Shc was also inhibited by phosphopeptide pY610. Thus, these peptide competition analyses suggest that the SH2 domain of Shc interacts with the residues surrounding tyrosine 612 of  $\beta c$  and the

**FIGURE IV.9**



**Phosphopeptide pY610 inhibits binding of the SH2 domain of Shc to tyrosine phosphorylated Aic2A.**

Factor deprived Ba/F3 cells were either left untreated as a control (C) or stimulated for 10 minutes with IL-3. (A) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100  $\mu$ M of the indicated phosphopeptides and SH2Shc-GST precipitates prepared. (B) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 1  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M of phosphopeptide pY610 and SH2Shc-GST precipitates prepared. The precipitates were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The positions of the molecular mass standards are shown and expressed in kDa and the position of Aic2A is indicated.

corresponding residues surrounding tyrosine 610 of Aic2A while the residues surrounding tyrosine 750/745 of  $\beta$ c/Aic2A may make some contribution to the interaction.

#### **IV.E.3 Binding of the Shc PTB domain to tyrosine phosphorylated $\beta$ c/Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 577 of $\beta$ c / tyrosine 575 of Aic2A.**

Given the observed association of the PTB domain of Shc with two phosphotyrosine-containing proteins, one of which was identified as  $\beta$ c in human cells, and by analogy Aic2A in murine cells, the site on  $\beta$ c/Aic2A which might be responsible for this interaction was investigated. PTB domains recognise phosphotyrosines in the motif NPXpY (Kavanaugh *et al.*, 1995; Songyang *et al.*, 1995; van der Geer and Pawson, 1995). Amino acids in the -5 position relative to the phosphotyrosine are also reported to be important for PTB domain recognition and aliphatic residues predominate in this region (Trub *et al.*, 1995; van der Geer *et al.*, 1995; Zhou *et al.*, 1995a). Tyrosine 577 of  $\beta$ c and tyrosine 575 of Aic2A lie in the motif NGPY which loosely conforms to this predicted PTB-binding consensus sequence. Therefore, phosphopeptides containing additional amino acids amino-terminal to the critical phosphotyrosine were synthesised based on sequences surrounding tyrosine 575, 610 and 745 of Aic2A but which have identical sequences amino-terminal to tyrosine 577 and 612 of  $\beta$ c and differ by 2 amino acids to tyrosine 750 of  $\beta$ c. These phosphopeptides were used in competition analyses and results are shown in Figures IV.10 (representative of two to three individual experiments) and IV.11 (representative of three separate experiments). The phosphopeptide pY575 completely inhibited the precipitation the 130-145 kDa tyrosine phosphorylated proteins by the PTBShc-GST fusion protein in TF-1 cells (Fig. IV.10 A) and in Ba/F3 cells (Fig. IV.11 A). Titration of phosphopeptide pY575 indicated that at only 1  $\mu$ M peptide the association between PTBShc-GST and tyrosine phosphorylated proteins were almost completely blocked in TF-1 cells (Fig. IV.10 B) and in Ba/F3 cells (Fig. IV.11 B).

The 145 kDa polyphosphate-5-phosphatase SHIP, has also been shown to interact with the PTB domain of Shc (Lioubin *et al.*, 1996). In the studies presented here, SHIP has been shown to be present in the PTBShc-GST precipitations (Fig. IV.4 A) and also appears to be competed by the phosphopeptide pY575 (Fig. IV.10 A). Therefore, in order to specifically

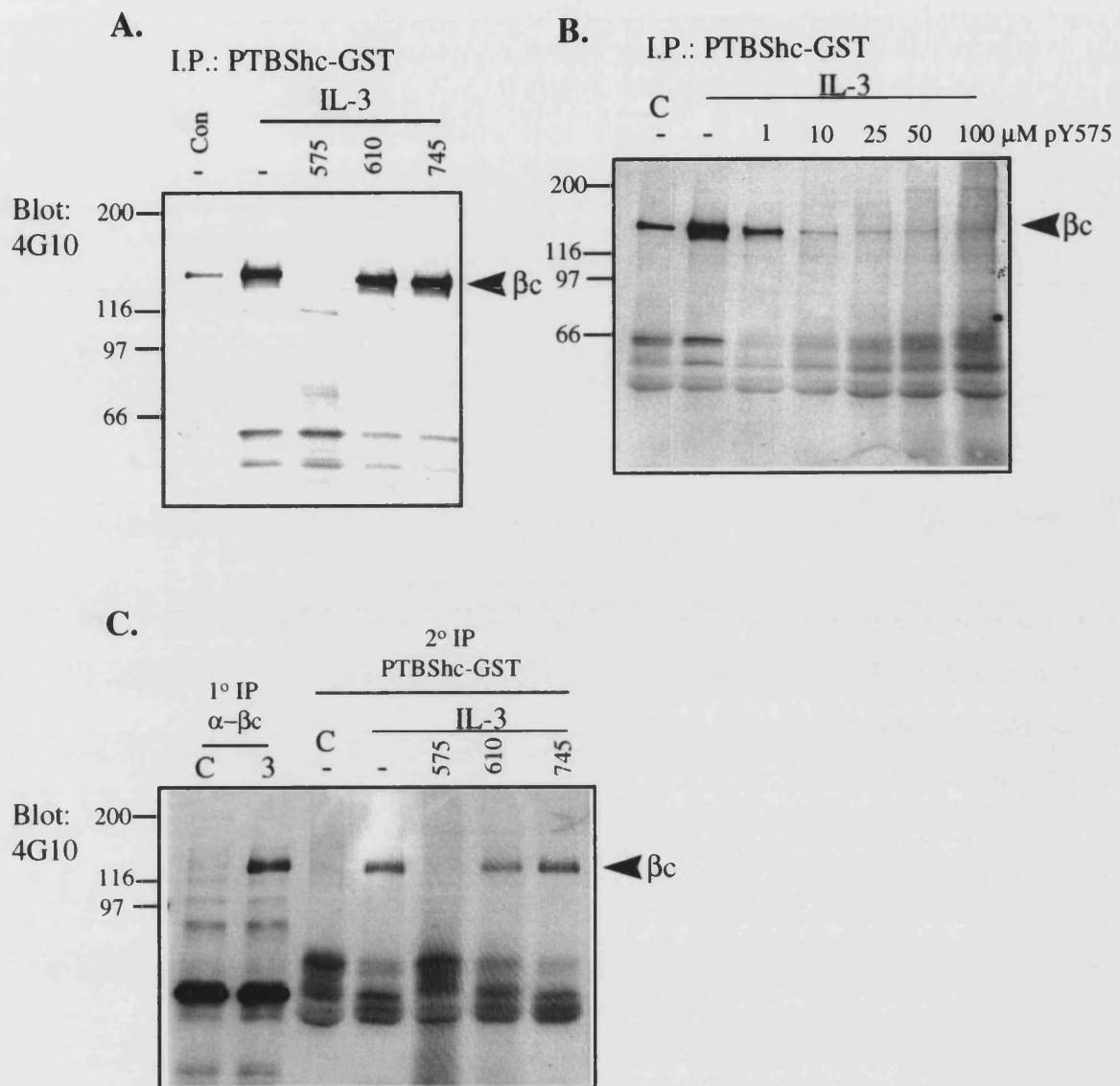


#### **FIGURE IV.10**

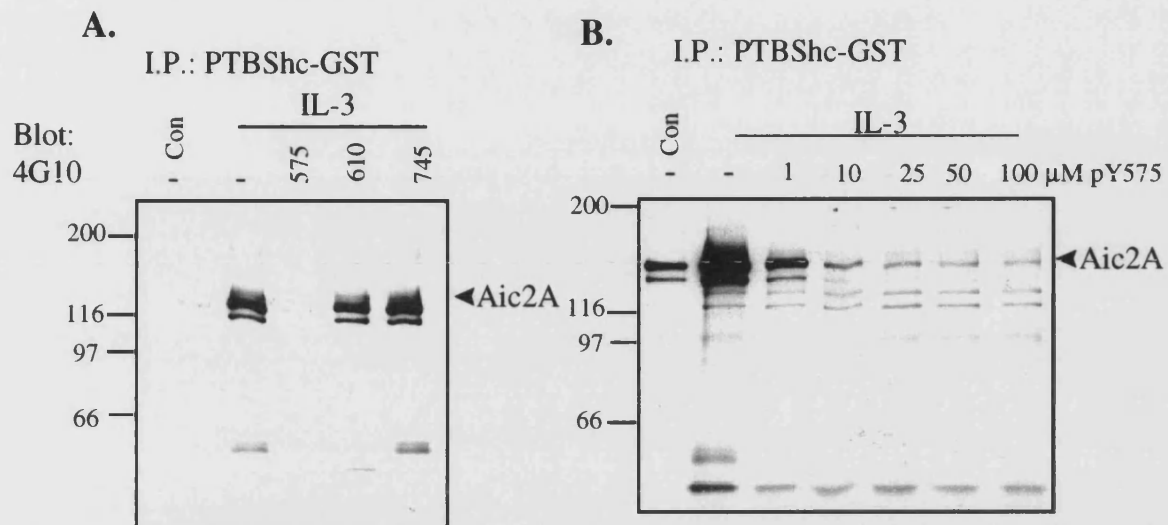
##### **Phosphopeptide pY575 inhibits binding of the PTB domain of Shc to tyrosine phosphorylated $\beta$ c.**

TF-1 cells were factor deprived and left untreated as a control or stimulated with IL-3. (A) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100  $\mu$ M of the indicated phosphopeptides and PTBShc-GST precipitates prepared. (B) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 1  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M of phosphopeptide pY575 and PTBShc-GST precipitates prepared. (C) Cell extracts from the equivalent of  $2 \times 10^7$  cells/sample were precipitated with 20  $\mu$ g monoclonal anti- $\beta$ c antibody. Primary anti- $\beta$ c precipitates were eluted and denatured by boiling in SDS sample buffer and 1/10 of the sample reserved for the primary immunoprecipitation sample. Secondary precipitations were prepared with the PTBShc-GST fusion protein. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The molecular mass standards are expressed in kDa and the position of  $\beta$ c is indicated.

**FIGURE IV.10**



**FIGURE IV.11**



**Phosphopeptide pY575 inhibits binding of the PTB domain of Shc to tyrosine phosphorylated Aic2A.**

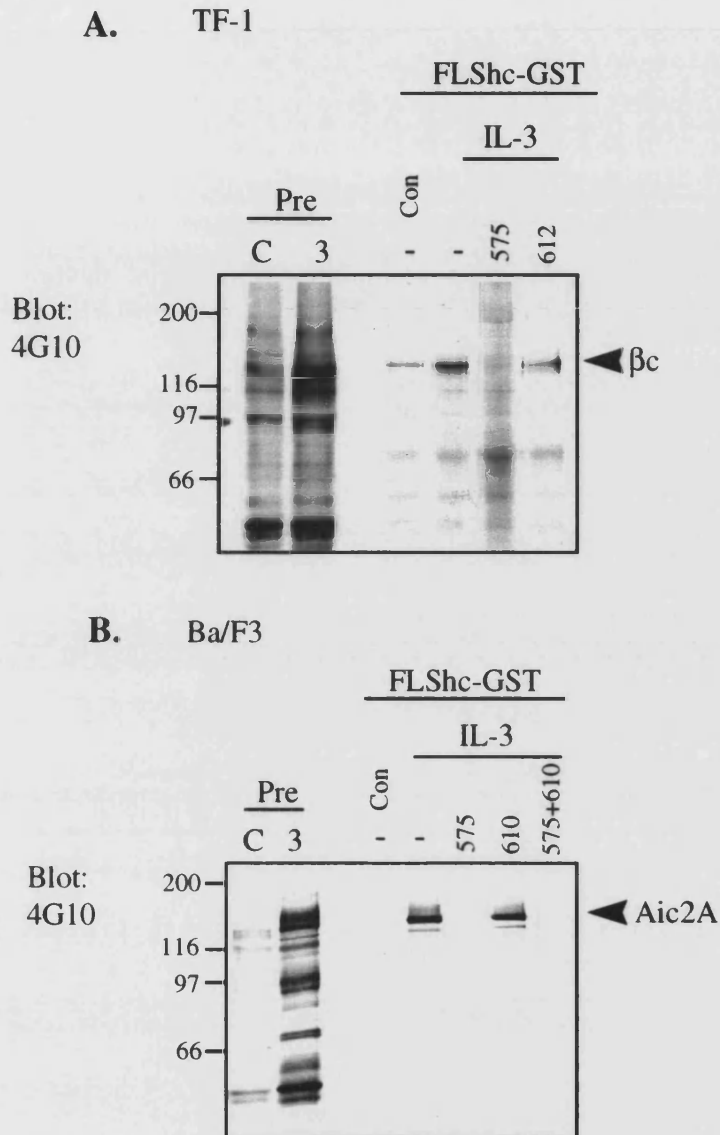
Ba/F3 cells were factor deprived and left untreated as a control or stimulated with IL-3. (A) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100 μM of the indicated phosphopeptides and PTBShc-GST precipitates prepared. (B) Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 1 μM, 10 μM, 25 μM, 50 μM, or 100 μM of phosphopeptide pY575 and PTBShc-GST precipitates prepared. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. The molecular mass standards are shown and expressed in kDa and the position of Aic2A is indicated.

examine competition for  $\beta c$  binding, sequential immunoprecipitation analyses were performed in TF-1 cells to assess whether phosphopeptide pY575 was blocking the PTB domain of Shc binding specifically to tyrosine phosphorylated  $\beta c$ . TF-1 cell extracts were initially immunoprecipitated with the anti- $\beta c$  monoclonal antibody. The precipitated material was diluted and incubated with the appropriate phosphopeptide. A secondary precipitation with the PTBShc-GST fusion protein that had been preincubated with the phosphopeptide was then performed. The results are shown in Figure IV.10 C. Tyrosine phosphorylated  $\beta c$  from cells treated with IL-3 was precipitated in the primary anti- $\beta c$  immunoprecipitation. The secondary precipitation using the PTBShc-GST fusion protein preincubated without phosphopeptide or with phosphopeptide pY610 and pY745 resulted in re-precipitation of tyrosine phosphorylated  $\beta c$  from IL-3 stimulated cell extracts. However, incubation of the phosphopeptide pY575 with the PTBShc-GST fusion protein was able to completely block re-precipitation of tyrosine phosphorylated  $\beta c$  from IL-3-stimulated cell extracts. Thus, taken together, these results suggest that the PTB domain of Shc interacts with the amino acids surrounding tyrosine 577 of  $\beta c$  and tyrosine 575 of Aic2A. These results are consistent with results of Pratt *et al.* (1996) where tyrosine 577 was shown to be sufficient for the binding of the PTB domain of Shc to  $\beta c$  in a non-ligand-dependent system.

#### **IV.E.4 Binding of FL Shc to tyrosine phosphorylated $\beta c$ /Aic2A is inhibited by a phosphotyrosine containing peptide based on sequences surrounding tyrosine 577 of $\beta c$ / tyrosine 575 of Aic2A.**

Having shown that both the SH2 and PTB domains of Shc interact with residues surrounding distinct tyrosines within  $\beta c$ /Aic2A, albeit with apparently differing affinities, it was next investigated whether one of these domains played a dominant role in mediating the interaction of Shc with  $\beta c$ /Aic2A. To test this, phosphopeptide competition analyses were performed using the FLShc-GST fusion protein. These results are shown in Figure IV.12 and are representative of two to three separate experiments. At 100  $\mu M$ , the phosphopeptide representing residues surrounding tyrosine 612 of  $\beta c$  was unable to inhibit the precipitation of tyrosine phosphorylated  $\beta c$  by the FLShc-GST fusion protein in TF-1 cells, whereas

**FIGURE IV.12**



**Phosphopeptide pY575 inhibits binding of FL Shc to tyrosine phosphorylated βc/Aic2A.**

Factor deprived (A) TF-1 cells and (B) Ba/F3 cells were either left untreated as a control (C) or stimulated with IL-3 (3). Cell extracts from the equivalent of  $1 \times 10^7$  cells were incubated in the absence (-) or the presence of 100 μM of the indicated phosphopeptides and FLShc-GST precipitates prepared. The samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 antibodies. The positions of the molecular mass standards are shown and expressed in kDa and the positions of βc and Aic2A are indicated.

phosphopeptide pY575 was able to inhibit this association (Fig. IV.12 A). In Ba/F3 cells, the phosphopeptide representing residues surrounding tyrosine 610 of Aic2A was unable to inhibit the precipitation of tyrosine phosphorylated Aic2A by the FLShc-GST fusion proteins whereas phosphopeptide pY575 completely inhibited this association. Additionally, it also appears that phosphopeptide pY577 was also capable of inhibiting the precipitation of tyrosine phosphorylated SHIP.

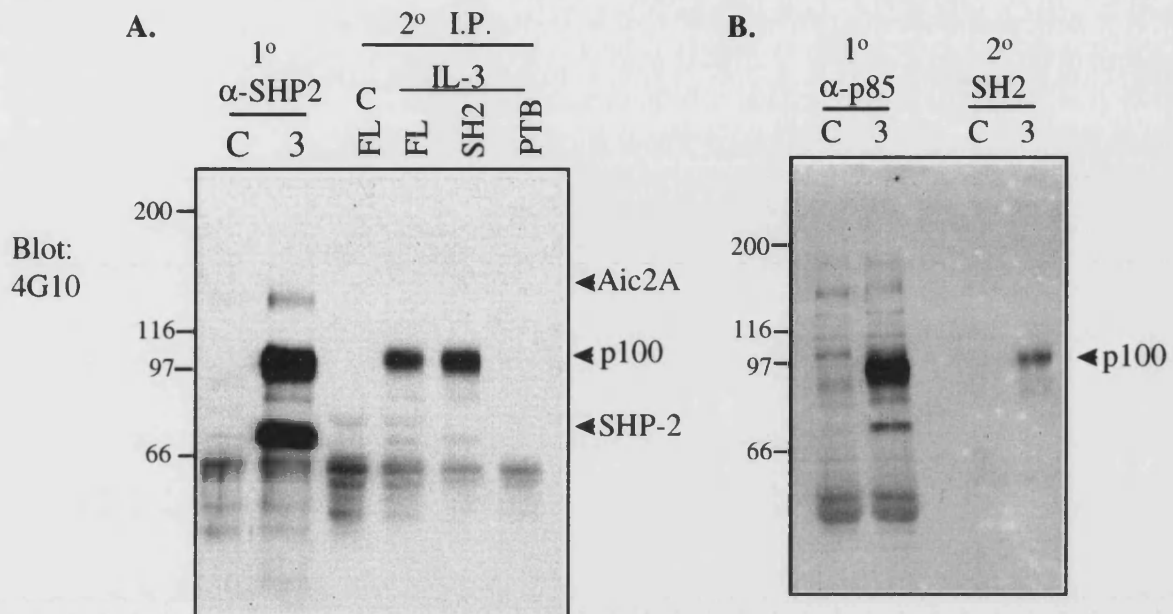
Taken together, these peptide competition analyses suggest that Shc interacts with tyrosine phosphorylated  $\beta$ c/Aic2A primarily via its PTB domain and the sequences surrounding tyrosine 577 of  $\beta$ c/ 575 of Aic2A, whereas the Shc SH2 domain interacts with residues surrounding tyrosine 612 of  $\beta$ c/ 610 of Aic2A with lower affinity and therefore may play a secondary or stabilising role.

#### **IV.F Interaction of Shc with a tyrosine phosphorylated 100 kDa protein in Ba/F3 cells.**

In Shc-GST precipitates from IL-3 stimulated Ba/F3 cells, a tyrosine phosphorylated 100 kDa protein was precipitated by the FLShc- and SH2Shc- GST fusion proteins, but not by the PTBShc-GST fusion protein (Fig. IV.3 B). A tyrosine phosphorylated 100 kDa protein has also been shown to be precipitated by Grb2-GST, anti-p85 PI3-K antibodies, the isolated SH2 domains of p85 and anti-SHP-2 antibodies in IL-3 stimulated FD-5 cells (Welham *et al.*, 1994b). This 100 kDa protein has now been shown to directly interact with the p85 subunit of PI3-K and SHP-2 (Craddock and Welham, 1997) and has been recently cloned and has been called Gab2 (Gu *et al.*, 1998) (see section III.E).

To determine if the Shc-GST fusion proteins could also directly bind to this tyrosine phosphorylated p100 protein which associates with SHP-2, sequential immunoprecipitations were performed in Ba/F3 cells which had either been left untreated as a control (C) or stimulated with IL-3 for 10 minute (3). Cell extracts were initially immunoprecipitated with anti-SHP-2 antibodies. The precipitated material was boiled and denatured with SDS and  $\beta$ -mercaptoethanol and the resulting extracts reprecipitated with the FLShc-, SH2Shc-, or PTBShc- GST fusion proteins. As would be expected, the primary anti-SHP-2 antibodies immunoprecipitated a major tyrosine phosphorylated p100 protein from IL-3 stimulated Ba/F3

**FIGURE IV.13**



**The SH2, but not the PTB, domain of Shc binds directly to p100.**

Factor deprived Ba/F3 cells were either left untreated as a control (C) or stimulated with IL-3 (3). (A) Cell extracts from the equivalent of  $2 \times 10^7$  cells/sample were precipitated with 1  $\mu$ g anti-SHP-2 antibody. Primary anti-SHP-2 precipitates were eluted and denatured by boiling in SDS sample buffer and 1/10 of the sample reserved for the primary immunoprecipitation sample. After dilution, the samples were re-precipitated with 10  $\mu$ g of either the FL-, PTB- or SH2- Shc-GST fusion proteins. (B) Cell extracts from the equivalent of  $2 \times 10^7$  cells were precipitated with anti-p85 antibodies. The primary precipitation was eluted, denatured and diluted before a secondary precipitation with 10  $\mu$ g SH2Shc-GST fusion protein. All samples were resolved by SDS-PAGE through 7.5% acrylamide gels and immunoblotted with 4G10 antibodies. The positions of Aic2A, p100 and SHP-2 are indicated and the molecular mass standards are shown and expressed in kDa.

cells (Fig. IV.13 A). A faint band of around 135 kDa was also precipitated. This is most likely Aic2A, as it has already been shown that SHP-2 associates with  $\beta c$  in human cells (see Chapter III). The secondary precipitation with the FLShc- and the SH2Shc- GST fusion proteins re-precipitated the tyrosine phosphorylated p100 protein from IL-3 stimulated cells, but the PTBShc-GST fusion protein did not, paralleling the results seen initially in the direct FLShc-, PTBShc- and SH2Shc- GST precipitations (Fig. IV.3 B). The tyrosine phosphorylated 135 kDa Aic2A protein was not observed to be re-precipitated by the Shc-GST fusion proteins as would be expected. This was probably due to detection limit problems as only a faint tyrosine phosphorylated 135 kDa band was observed in the initial anti-SHP-2 immunoprecipitation. Alternatively, SHP-2 may not precipitate the same subset of tyrosine phosphorylated Aic2A that Shc is able to bind. These results were observed in two separate experiments and suggest that the SH2 domain of Shc can directly interact with the same tyrosine phosphorylated p100 protein precipitated by SHP-2 in IL-3 stimulated Ba/F3 cells.

To investigate whether the p100 protein precipitated by the Shc SH2 domain was the same protein observed to interact with the p85 subunit of PI3-K (Craddock and Welham, 1997), sequential immunoprecipitations in which p100 was first precipitated by anti-p85 antibodies and then reprecipitated by the ShcSH2-GST fusion protein were performed (Fig. IV.13 B). A tyrosine phosphorylated 100 kDa protein was precipitated by anti-p85 antibodies from Ba/F3 cells which had been stimulated for 10 minutes with IL-3 (3). This protein was reprecipitated by the SH2Shc-GST fusion protein suggesting that Shc can bind directly via its SH2 domain to the same tyrosine phosphorylated protein co-precipitated by anti-p85 antibodies (these results were observed in two individual experiments). Thus it appears that Shc can associate, via its SH2 domain, with the same p100 protein which directly associates with both SHP-2 and the p85 subunit of PI3-K and is therefore likely to be Gab2.



#### **IV.G Discussion.**

The data presented here demonstrate that following IL-3 stimulation, the adaptor protein Shc can inducibly associate with  $\beta$ c/Aic2A, via both its PTB and SH2 domains, with the inositol polyphosphate-5-phosphatase SHIP via its PTB domain, and with a murine 100 kDa protein and human 120 kDa protein via its SH2 domain. Mapping studies, based on phosphopeptide competition analyses, suggest residues surrounding tyrosine 612/610 of  $\beta$ c/Aic2A are the predominant site of Shc SH2 interaction and residues surrounding tyrosine 577/575 of  $\beta$ c/Aic2A are the predominant site for Shc PTB-mediated association. These results extend previous studies which had reported GM-CSF-induced association of Shc with  $\beta$ c (Lanfranccone *et al.*, 1995) but had not mapped the site of association. Also, in COS cells, non-ligand-dependent, JAK2 phosphorylation of  $\beta$ c at tyrosine 577 had been shown to be sufficient for the binding of the PTB domain of Shc to  $\beta$ c (Pratt *et al.*, 1996). This is now shown to be an IL-3-regulated event, which is important, given that there is evidence which suggests that GM-CSF and IL-3 can regulate signalling pathways differently (Scheid *et al.*, 1995).

Association of Shc with transmembrane receptors is believed to be crucial in activation of the ras-MAP kinase pathway (Bonfini *et al.*, 1996). The SH2 domain of Shc has been shown to associate with transmembrane receptors, such as the EGFR (Pelicci *et al.*, 1992, Blaikie *et al.*, 1994), the PDGFR (Yokotoe *et al.*, 1994) and with gp130 in response to IL-6 (Gioradano *et al.*, 1997) but the precise binding sites have not been determined. The sequence surrounding tyrosine 612 of  $\beta$ c (LEYLCLP) and tyrosine 610 of Aic2A (LEYMCLP) broadly conform to the predicted consensus recognition sequence for the Shc SH2 domain of pY[I/E/Y/L]x[I/L/M] (Songyang *et al.*, 1994) and based on the results presented here, it would appear tyrosine 612/610 of  $\beta$ c/Aic2A is the primary ShcSH2-binding site of reasonable affinity. A partial inhibition of precipitation of  $\beta$ c/Aic2A with the SH2Shc-GST fusion protein by phosphopeptide pY750/pY745 was also observed. The sequence surrounding tyrosine 750/745 in  $\beta$ c/Aic2A is YVEL which also loosely conforms to the Shc SH2 domain consensus binding motif. Work by Inhorn *et al.* (1995) has shown a reduction in Shc tyrosine phosphorylation in response to GM-CSF when tyrosine 750 of  $\beta$ c is mutated to phenylalanine

but binding of Shc to this residue was not investigated. Interestingly, Pratt *et al.* (1996) did not see an association of  $\beta c$  with the SH2 domain of Shc using a SH2-CH-GST fusion protein. However, they used a non-ligand dependent system relying solely on transfected JAK2 for phosphorylation of transfected  $\beta c$ , so perhaps an additional kinase is required to associate with the receptor to phosphorylate tyrosine 612.

In response to IL-3, the PTB domain of Shc was also shown to interact directly with tyrosine phosphorylated  $\beta c$  at sequences surrounding tyrosine 577. Other receptors, including the EGFR (Blaikie *et al.*, 1994) and the NGFR (van der Geer *et al.*, 1995; Dikic *et al.*, 1995) have previously been shown to interact with the PTB domain of Shc. Data derived from examining the binding of the Shc PTB domain to sequence motifs derived from growth factor receptors and oncoproteins has defined the minimal Shc PTB binding motif as N-X-X-pY and suggest a high affinity motif as hydrophobic residue-(D/E)-N-X-X-pY-(W/F) (Lamiet *et al.*, 1996). The only residues within the  $\beta c$ /Aic2A cytoplasmic domain that fits such a recognition motif are surrounding tyrosine 577/575 (SFDFNGPpYLGP). Mutation of tyrosine 577, in  $\beta c$ , to phenylalanine is reported to lead to abolition of detectable tyrosine phosphorylated Shc in response to GM-CSF (Durstin *et al.*, 1996). However, this study did not address the direct binding of Shc to tyrosine 577 of  $\beta c$ .

The interaction of the PTB domain with residues surrounding tyrosine 577/575 of  $\beta c$ /Aic2A appears to be of higher affinity when compared to the association of the SH2 domain of Shc with residues surrounding tyrosine 612/610 of  $\beta c$ /Aic2A, as only 1  $\mu$ M pY575 was sufficient to inhibit the association of PTBShc-GST with  $\beta c$ /Aic2A (Fig. IV.10 B and IV.11 B), whereas 25  $\mu$ M pY612 was required to inhibit the association of SH2Shc-GST with  $\beta c$ /Aic2A (Fig. IV.8 B and IV.9 B). These findings are consistent with reports that the binding affinities of the Shc PTB domain to tyrosine phosphorylated peptides are as much as 100 times greater than those of the Shc SH2 domain (Kavanaugh *et al.*, 1995; Zhou *et al.*, 1996b). In response to EGF, the PTB domain of Shc is also reported to bind with higher affinity to the EGF receptor when compared to the affinity of the SH2 domain (Sakaguchi *et al.*, 1998). Additionally here, only phosphopeptide pY575 was able to inhibit the IL-3-induced association of FL Shc with  $\beta c$ /Aic2A and not phosphopeptide pY612/610 (Fig. IV.12). This would suggest that Shc primarily binds via its PTB domain to the sequences surrounding

tyrosine 577/575 of  $\beta c/Aic2A$ . It is possible that after this association, Shc may then bind via its SH2 domain to the sequences surrounding tyrosine 612/610. This secondary binding may induce a functionally important conformational change important for mediating downstream signalling events, perhaps by making tyrosine 317 accessible to the SH2 domain of Grb2.

Shc is one of the primary targets of IL-3-induced tyrosine phosphorylation (Welham *et al.*, 1994a). The majority of tyrosine phosphorylated Shc resides in the cytosol and it is still not clear whether Shc needs to be recruited to  $\beta c$  in order for it to become tyrosine phosphorylated. It has previously been shown that Ba/F3 cells expressing a tyrosine to phenylalanine mutation at tyrosine 577 of  $\beta c$  are defective in GM-CSF-induced phosphorylation of Shc but the binding of Shc to  $\beta c$  was not examined (Durstin *et al.*, 1996). Itoh *et al.* (1996) and Okuda *et al.* (1997) also show that tyrosine 577 is essential and sufficient for Shc phosphorylation but again the association of Shc with  $\beta c$  was not examined. In mutants expressing a tyrosine to phenylalanine mutation at 612, normal Shc phosphorylation was reported (Durstin *et al.*, 1996) and in mutants expressing only tyrosine 612, with all others tyrosines mutated to phenylalanine, no phosphorylation of Shc was observed (Okuda *et al.*, 1997). Taken together with the results presented here, these data suggest that Shc does not need to bind to residues surrounding tyrosine 612/610 in order to become tyrosine phosphorylated. Rather, the initial binding of the Shc PTB domain to tyrosine 577/575 of  $\beta c/Aic2A$  may be sufficient to localise Shc so that it can be tyrosine phosphorylated, if this is indeed required. However, one cannot exclude the possibility that the tyrosine 577 to phenylalanine mutants fail to recruit/activate a critical kinase or kinases required for Shc phosphorylation, thus explaining the lack of Shc phosphorylation in such mutant receptor expressing cells.

The PTB domain of Shc also appeared to be directly interacting with tyrosine phosphorylated proteins of 130-145 kDa which were identified as the inositol polyphosphate-5-phosphatase SHIP in Ba/F3 and TF-1 cells (section IV.D). Both the 130 and 140 kDa proteins in IL-3 stimulated Ba/F3 cells immunoreacted with anti-SHIP antibodies. Kavanaugh *et al.* (1996) also showed the PTB domain of Shc interacting with tyrosine phosphorylated 130 and 145 kDa proteins which were determined to be alternative products of the same gene. Additionally, antiserum generated against the SH2 domain of SHIP has been shown to

recognise the 145-, 135-, 125-, and 110 kDa proteins in many haemopoietic cell lines (Liu *et al.*, 1997 a, b) and some of the lower molecular weight proteins have recently been identified as C-terminal truncations of the full-length SHIP protein (Damen *et al.* 1998). Therefore, both the tyrosine phosphorylated 130 and 140 kDa proteins interacting with the PTB domain of Shc are most likely SHIP and a C-terminally truncated full length SHIP protein.

SHIP has been shown to interact with Shc via two mechanisms: i) the PTB domain of Shc can bind NPXpY motifs of SHIP and ii) the SH2 domain of SHIP can bind via tyrosine 317 of Shc *in vitro* (Liu *et al.*, 1997a). The PTB domain of Shc has been shown to be necessary and sufficient for its association with tyrosine phosphorylated SHIP during T cell receptor signalling and the carboxyl terminal NPXpY motifs of SHIP appear to be required for the *in vivo* association of Shc with SHIP (Lamkin *et al.*, 1997; Liu *et al.*, 1997a). However, there are conflicting reports on the association of the SH2 domain of SHIP with Shc. One study has reported that a functional SHIP SH2 domain was also required for association with Shc as SHIP mutants lacking a functional SH2 domain did not precipitate Shc and were not detectably tyrosine phosphorylated (Liu *et al.*, 1997a). They suggest that the SH2 domain of SHIP is required to bind to a tyrosine kinase directly or bind to Shc via residues surrounding tyrosine 317 to be translocated to the vicinity of a tyrosine kinase. The subsequent phosphorylation of SHIP would allow the PTB domain of Shc to bind to SHIP. However, this may be an indirect effect as mutation of the SH2 domain of SHIP abrogated SHIP tyrosine phosphorylation in these experiments and thus would preclude the binding of the Shc PTB domain to SHIP. This alternate interpretation is supported by Lamkin *et al.* (1997) who observed that the SH2 domain of SHIP was dispensable for Shc-SHIP interaction as a Shc construct containing a mutant PTB domain failed to interact with SHIP. In addition, a CH-SH2 domain construct (which contains tyrosine 317) also failed to co-precipitate SHIP (Lamkin *et al.*, 1997). This does not rule out the possibility that the SH2 domain of SHIP interacts with tyrosine 317 of Shc, but may suggest that this interaction is not of high affinity or is a secondary interaction.

Phosphopeptide pY575 not only inhibited PTBShc- (Fig. IV.10 and IV.11) and FLShc-GST (Fig. IV.12) from interacting with the tyrosine phosphorylated IL-3 receptor, but also inhibited the precipitation of tyrosine phosphorylated SHIP. Binding of pY575 to the Shc PTB

domain would prevent tyrosine phosphorylated SHIP from interacting with the PTB domain of Shc. Since pY575 inhibited both the PTB domain alone and FL Shc from precipitating tyrosine phosphorylated Shc, this would suggest that tyrosine phosphorylated SHIP primarily interacts with the PTB domain of Shc and that the interaction with the SH2 domain of SHIP and tyrosine 317 of Shc is a secondary or lower affinity interaction. SHP-2 has also been shown to associate with SHIP following IL-3 stimulation. The association has been shown *in vitro* to occur through a direct interaction of the SH2 domain of SHIP with a pYXN(I/V) motif within SHP-2 (Liu *et al.*, 1997b). Thus, prevention of SHIP binding to the PTB domain of FL Shc by pY575 may alternatively weaken the affinity of SHIP for Shc and result in the binding of SHIP to SHP-2. This could subsequently lead to the dephosphorylation of SHIP by SHP-2.

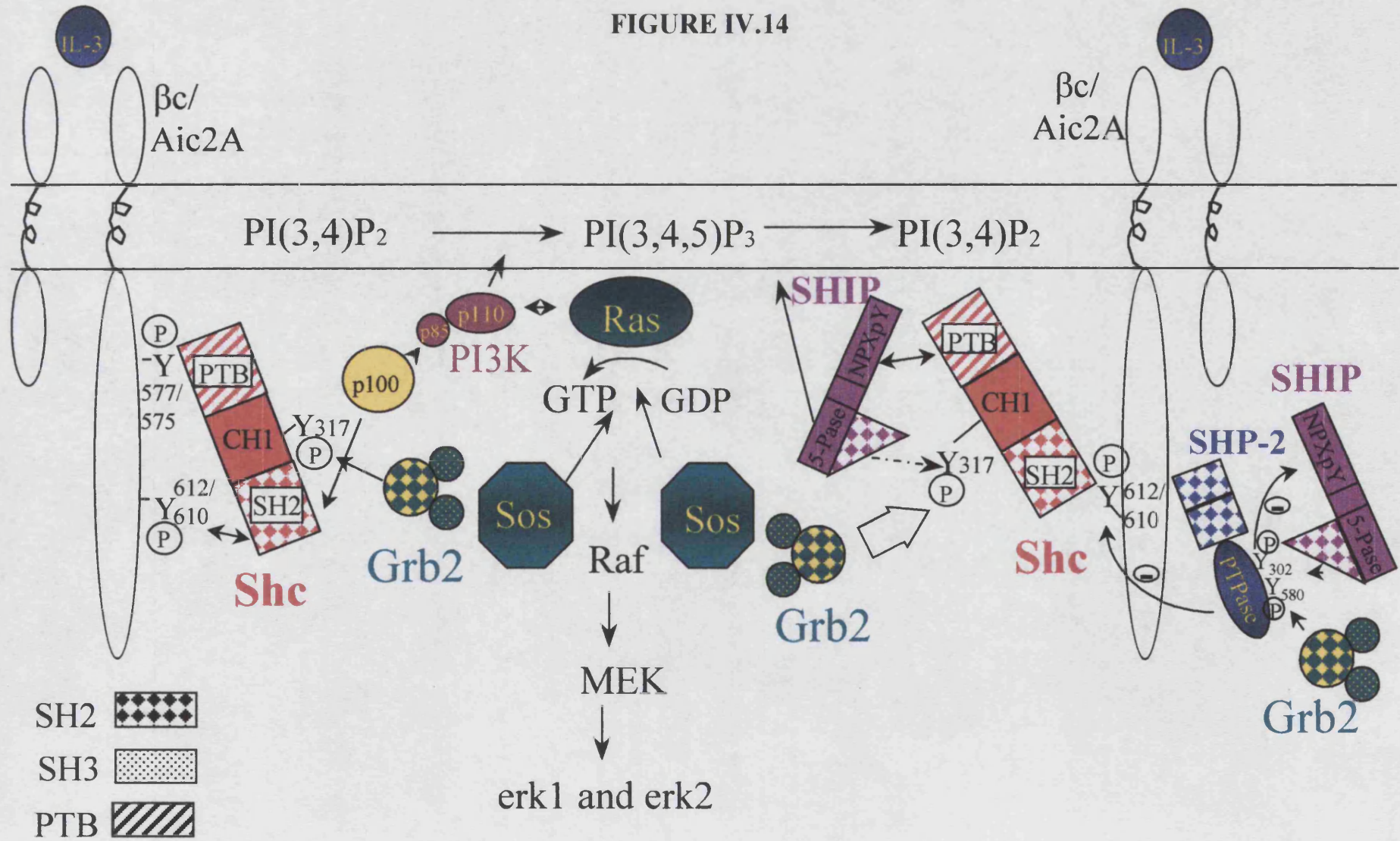
SHIP has been shown to play a negative role in growth factor signalling. Overexpression of SHIP has been shown to induce an apoptotic effect in DA-ER cells which is mediated by the SH2 domain of SHIP (Liu *et al.*, 1997a) and retroviral expression of SHIP has been shown to result in inhibition of cell growth in response to M-CSF and IL-3 in FD cells (Lioubin *et al.*, 1996). In B cells, co-clustering of the B cell receptor and FcγR, an early event linked to the down-regulation of proliferation induced by antigen receptor stimulation, is required for optimal SHIP tyrosine phosphorylation and its association with Shc (Chacko *et al.*, 1996) and it has been proposed that SHIP may function to inhibit early activation events like calcium influx (Ono *et al.*, 1996). SHIP has inositol polyphosphate-5-phosphatase activity and can hydrolyse PI(3,4,5)P<sub>3</sub>, the primary product of PI3K, to PI(3,4)P<sub>2</sub> (Damen *et al.*, 1996; Lioubin *et al.*, 1996). Thus, localisation of SHIP to the vicinity of its lipid substrates could lead to down-regulation of PI3-K-induced proliferation and survival signals (Yao and Cooper, 1995). Alternatively, SHIP may act as a negative regulator by competing with Grb2 for binding to SHP-2 or Shc and thereby down-regulate the Ras/MAP kinase pathway.

The SH2 domain of Shc also appeared to interact with a 120 kDa protein in IL-3-stimulated TF-1 cells and directly interact with a tyrosine phosphorylated 100 kDa protein following IL-3 stimulation of Ba/F3 cells. The precipitation of both p100 and p120 by the SH2Shc-GST fusion protein were also inhibited by phosphopeptides pY610 (Fig. IV.9) and pY612 (Fig. IV.8) respectively. In Ba/F3 cells, this protein appeared to be the same 100 kDa protein which had previously been shown to directly interact with both the SH2 domains of the

p85 subunit of PI3-K and SHP-2 (Fig. IV.13) (Craddock and Welham, 1997). It has now been shown by Gu *et al.* (1998) that this 100 kDa protein is Gab2 and the results presented here are consistent with their findings that Gab2 associates with SHP-2, Shc and the p85 subunit of PI3-K. Since Gab2 was found to be expressed in many haemopoietic cell lines (Gu *et al.*, 1998), it is likely that the 120 kDa protein associated with the SH2 domain of Shc in TF-1 cells is the human Gab2 protein. It has been previously proposed that direct binding of SHP-2 to the IL-3 receptor  $\beta$  subunit would allow for recruitment of a p100:p85(PI3K) complex and hence translocate PI3-K to the vicinity of its lipid substrates (Craddock and Welham, 1997). Thus, Shc may function in a similar manner, allowing translocation of the p100:p85(PI3-K) complex to the plasma membrane. Additionally, the p110 catalytic subunit of PI3-K has been shown to directly interact with Ras (Rodriguez-Viciana *et al.*, 1994); however, it is not clear whether PI3-K acts as an effector or regulator of Ras. While evidence strongly suggests that PI3-K can stimulate Ras-dependent cellular processes, importantly activation of the MAP kinases erk1 and erk2 (Ferby *et al.*, 1994; Welsh *et al.*, 1994; Hu *et al.*, 1995; Jascur *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Sharma *et al.*, 1998), other data also suggests that Ras can regulate PI3-K (Rodriguez-Viciana *et al.*, 1994; Kauffmann-Zeh *et al.*, 1997). Therefore, Shc may function in translocating a Gab2:p85(PI3-K) complex to the plasma membrane transmitting signals resulting in conversion of PI(3,4)P<sub>2</sub> to PI(3,4,5)P<sub>3</sub>, or resulting in Ras/MAP kinase regulation.

Based on the observations presented here, the following model can be proposed (see Fig. IV.14). The primary association between  $\beta$ c/Aic2A and Shc is mediated by binding of the PTB domain of Shc with tyrosine 577/575 of  $\beta$ c/Aic2A, which may lead to phosphorylation of tyrosine 317 of Shc, possibly by JAK2. The subsequent binding of the SH2 domain of Shc to tyrosine 612/610 of  $\beta$ c/Aic2A may then induce a conformational change in Shc, exposing phosphotyrosine 317 to which the SH2 domain of Grb2 could then bind, thereby leading to the localisation of Sos to the plasma membrane where it could activate Ras. Alternatively, the binding of the PTB domain alone to  $\beta$ c may be sufficient for the binding of the SH2 domain of Grb2 to tyrosine 317 of Shc. Shc may also function in localising SHIP to the plasma membrane. The SH2 domain of SHIP has been shown to associate with Shc via tyrosine 317 and the NPxY motifs within SHIP associate with the PTB domain of Shc (Liu *et al.*, 1997a).

FIGURE IV.14



Model: the role of Shc in mediating IL-3 signalling pathways

Therefore, the association of the SH2 domain of Shc with  $\beta$ c/Aic2A could function in localising SHIP to the plasma membrane. SHIP may also be localised to the membrane by association with SHP-2, as SHP-2 can also bind to residues surrounding tyrosine 612/610 of  $\beta$ c/Aic2A and SHIP has recently been shown to interact via its SH2 domain to one of the pYXN(I/V) motifs within SHP-2 (Liu *et al.*, 1997b). The localisation of SHIP to the membrane could function in the down-regulation of the PI3-K pathways as SHIP has the ability to dephosphorylate the primary product of PI3-K, PI(3,4,5)P<sub>3</sub>, to PI(3,4)P<sub>2</sub>. Alternatively, its binding to the Grb2 SH2 binding sites on Shc and SHP-2 could down-regulate the Ras/MAP kinase pathway. Finally, Shc can also bind, via its SH2 domain, to p100/Gab2. Therefore, binding of the Shc PTB domain to  $\beta$ c/Aic2A may function in localising p100 and hence PI3-K to the plasma membrane where it could catalyse the conversion of PI(3,4)P<sub>2</sub> to PI(3,4,5)P<sub>3</sub> or be involved as an effector or regulator of Ras.

The data presented here suggest that Shc may play a complex role in integrating intracellular signalling pathways from the IL-3 receptor (see Fig. IV.14). Evidence has been provided that Shc can, in an IL-3-dependent manner, directly interact with  $\beta$ c/Aic2A via its PTB domain and tyrosine 577/575 of  $\beta$ c/Aic2A and/or via its SH2 domain with tyrosine 612/610 of  $\beta$ c/Aic2A. These interactions provide not only a mechanism for localising the Grb2-Sos complex to the plasma membrane and so to the vicinity of Ras, but also potentially translocate SHIP and a p100:PI3-K complex to the plasma membrane. The functional role of these interactions in IL-3 signalling events are the subject of the following chapter.



## **CHAPTER V**

### **Expression of Shc Mutants in Ba/F3 cells.**

### **V.A. Introduction and aims.**

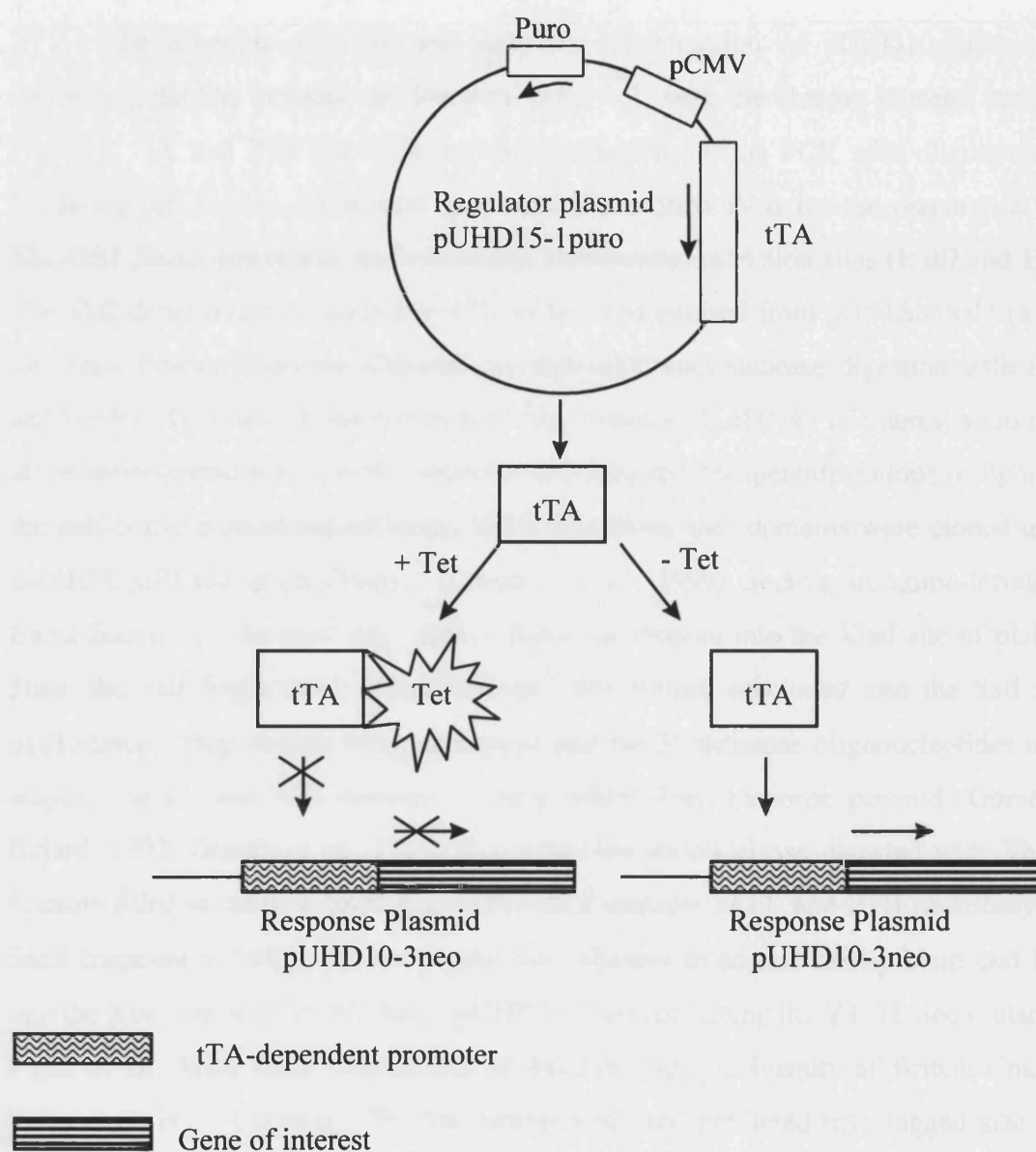
By examining the protein-protein interactions mediated by the various domains of Shc using GST fusion proteins, Shc has been shown to directly interact with the IL-3 receptor  $\beta$  subunit in both human ( $\beta c$ ) and mouse (Aic2A) cell lines in response to IL-3 (section IV.E). In addition, Shc has also been shown to bind via its PTB domain to SHIP (section IV.D) and via its SH2 domain to a tyrosine phosphorylated 100 kDa protein (section IV.F). Thus, Shc may function as an adaptor molecule, regulating not only the Ras/MAP kinase pathway but may also be involved in regulating other pathways involving PI3-K and lipid metabolism (see Fig. IV.14). To investigate the importance of Shc in integrating the complex network of IL-3-induced events, expression of various Shc mutants in the murine IL-3-dependent cell line, Ba/F3, and analyses of the functional consequences of their expression was next investigated. The effects of the Shc variants on IL-3-induced tyrosine phosphorylation of cellular substrates, activation of the MAP kinases erk1 and erk2 and proliferation were examined.

### **V.B. The tetracycline-regulated expression system.**

The choice of the appropriate *in vivo* expression system was deemed to be crucial to the potential success of these studies as expression of potentially dominant negative Shc mutants in IL-3-dependent cells may have detrimental effects on cell survival and proliferation, making derivation of stable clones constitutively expressing such variants difficult. Therefore, tight control of gene expression was essential. The expression system used in this study is a two vector system which utilises a tetracycline-sensitive regulator and is based on repression rather than induction (Gossen and Bujard, 1992; Gossen *et al.*, 1993) (see Fig. V.1). Classical regulatable expression systems rely on induction, rather than repression, often making it difficult to determine whether the effects observed are due to expression of the gene of interest or due to nonspecific effects of the drug used for induction (such as dexamethasone). The two vectors used in the tetracycline-regulated gene expression system are the regulator plasmid (pUHD15-1puro) and the response plasmid (pUHD10-3neo). The regulator plasmid encodes the tetracycline-sensitive transactivator, tTA, which is under control of the CMV promoter/enhancer, and followed

by the SV40 polyA signal and puromycin resistance marker for selection of transfected cells (Mui *et al.*, 1996). The response plasmid carries the neomycin resistance marker and has a tTA-dependent promoter upstream of the MCS for insertion of ones gene of interest. In this system, the presence of tetracycline represses the activity of the tetracycline-sensitive transactivator and prevents it binding to the promoter so the gene of interest from the response plasmid is not expressed. Removal of tetracycline allows tTA to bind to the promoter in the response plasmid and drive expression of the cDNA of interest.

FIGURE V.1



### Two vector tetracycline controlled regulatory gene expression system.

A tetracycline sensitive transactivator, tTA, is encoded by the regulator plasmid pUHD15-1puro. In the presence of tetracycline, the tTA is inactive and so is prevented from binding to the tTA-dependent promoter of the response plasmid, pUHD10-3neo, and the gene of interest is not expressed. Upon removal of tetracycline, tTA can bind to the promoter, resulting in expression of the gene.

### **V.C. Construction of pUHD10-3neo expression vectors.**

The plasmids used for the multi-step construction of pUHD10-3neo vectors, expressing the Shc variants, are listed in Table V.1, with the cloning strategy outlined in Fig. V.2. FL and PTB Shc domains were synthesised using PCR with oligonucleotides bordering the domains of interest, as outlined in section IV.B for the preparation of the Shc-GST fusion constructs, and containing appropriate restriction sites (BglII and EcoRI). The SH2 domain (amino acids 366-473) of Shc was excised from pGSTShcSH2 (a gift of Dr. Tony Pawson (Toronto, Canada)) by restriction endonuclease digestion with BamHI and EcoRI. To enable direct detection of Shc variants, all cDNAs of interest were tagged, at the amino-terminal end, with a sequence encoding the decapeptide epitope recognized by the anti-c-myc monoclonal antibody, 9E10. Therefore, the domains were cloned into the BamHI/EcoRI site of pSSBSmyc<sup>2</sup> (Craddock *et al.*, 1999) creating an amino-terminal in-frame fusion with the myc<sup>2</sup> tag. Before blunt end cloning into the XbaI site of pUHD10-3neo, the SalI fragment of SH2pSSBSmyc<sup>2</sup> was further subcloned into the SalI site of p1015Δstop. Stop codons were engineered into the 3' antisense oligonucleotides used to amplify the FL and PTB domains. The pUHD10-3neo response plasmid (Gossen and Bujard, 1992; Gossen *et al.*, 1993) was restriction endonuclease digested with XbaI and Klenow filled to create a blunt end. The SalI fragments of FL and PTB pSSBSmyc<sup>2</sup> and SacII fragment of SH2p1015Δstop were also Klenow filled and finally blunt end ligated into the XbaI site of pUHD10-3neo. pUHD10-3neo containing the Y317F Shc mutant, was a gift of Dr. Mike Gold (Department of Microbiology, University of British Columbia, Vancouver, B.C., Canada). The Shc amino acids and predicted myc tagged size of the expressed Shc variants are outlined in Table V.2.

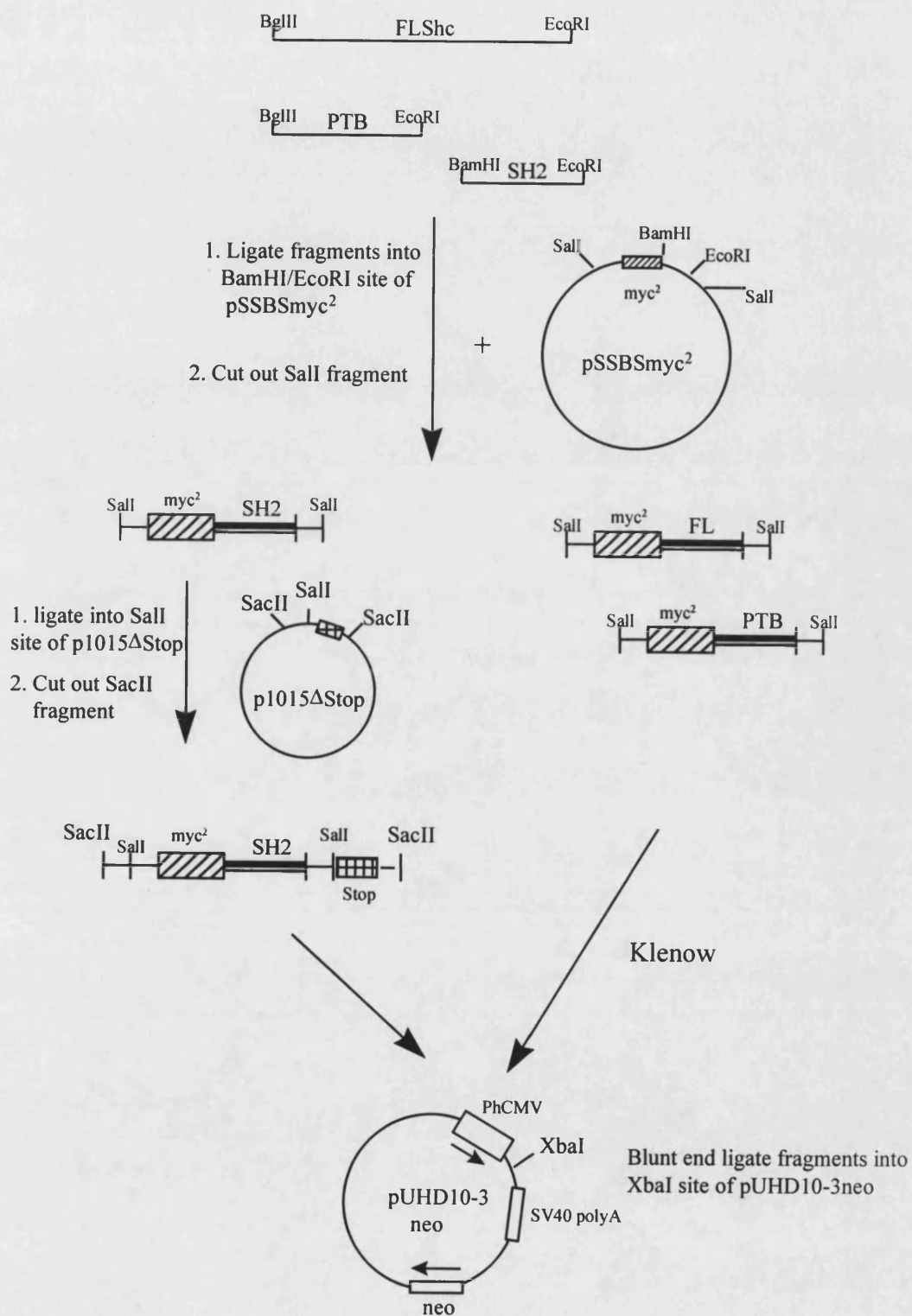
**FIGURE V.2****Cloning steps involved in construction of Shc pUHD10-3neo expression vectors.**

TABLE V.1

Plasmids used for construction of pUHD10-3 neo expression vectors

Plasmid	Features
pSSBS	pBluescript (Stratagene) + 2 SalI sites in MCS
pSSBSNmyc <sup>2</sup>	Vector containing tandem copies of the cDNA encoding the 10 amino acid myc tag epitope recognised by the monoclonal antibody, 9E10 (Evan <i>et al.</i> , 1985), with a short Gly-Ser linker following the second epitope, cloned into the NotI sites of the pBluescript based vector, pSSBS (Craddock <i>et al.</i> , 1999)
p1015Δstop	pBluescript based vector used to introduce in frame stop codons (gift of Dr. Paul Orban, EMBL, Heidelberg)
pUHD10-3neo	pBR322 based vector with a hCMV minimal promoter containing heptamerised tet-operators upstream in two different orientation as described by Gossen and Bujard (1992), multiple cloning site containing SacII, EcoRI, and XbaI sites, and a SV40 polyadenylation sequence downstream of the multiple cloning site. The neomycin resistance gene was cut out of PGKNeo with EcoRI, Klenow filled and blunt end ligated into the PvuII site of pUHD10-3 (gift of M. Gossen, Heidelberg)

TABLE V.2

Summary of Shc variants expressed from pUHD10-3neo

Construct	Shc amino acids	Predicted myc tagged size of expressed protein (kDa)
FLShc	1-473	58
Y317FShc	1-473	58
PTBShc	1-238	32
SH2Shc	366-473	18

**V.D. Expression of Shc mutants in IL-3 dependent Ba/F3 cells.**

To investigate the role of Shc in mediating IL-3-induced signalling events, various Shc mutants were expressed in IL-3-dependent Ba/F3 cells. Ba/F3 cells already expressing tTA from the plasmid pUHD15-1puro (Mui *et al.*, 1996) were electroporated with the response plasmid (pUHD10-3neo) encoding the amino-terminally myc-epitope-tagged Shc variants: full length Shc (FL); full length Shc with a mutation at tyrosine 317 (the Grb2 binding site) to phenylalanine (Y317F); the PTB domain alone (PTB); or the SH2 domain alone (SH2) (see section II.B.3). The Shc variants were tagged with a myc-epitope for easy detection of the expressed protein and for precipitation of these Shc variants from the cell. Transfectants were selected in the presence of tetracycline, G418 and puromycin and selected clones were assessed for inducible expression of the introduced cDNA by performing tetracycline removal time-course analyses (see section II.B.4). Initial screenings seemed to suggest that expression by the clones was very unstable and variable. After a couple of weeks, clones which initially had high inducible levels of expression showed only low constitutive levels. Continuing work by others in the lab eventually determined that the density at which the cells were set up was crucial to the level of induction observed. Reproducibly high levels of induction were observed when cells were cultured at a density of  $1 \times 10^5$  cells per ml or less. However, at densities which cells were routinely cultured, of approximately  $5 \times 10^5$  cells per ml, induction was more variable and often poor. This may be because if induction relies on removal of tetracycline from cells and the cells are growing more slowly due to their higher density, the levels of intracellular tetracycline may be depleted more slowly and induction may only occur at minimal levels. Therefore, cells were subsequently set up at  $1 \times 10^5$  cells per ml for selection and the following analyses of the clones.

For each Shc variant, at least 100 clones were screened for inducible expression of the various Shc mutants and two clones of each variant which showed low basal levels of expression in the presence of tetracycline and high inducible expression levels when tetracycline was removed were selected for further detailed analyses. Initial screenings involving extensive tetracycline removal time-courses showed expression occurring within 7 hours and maximal expression 16-24 hours after removal of tetracycline (results not shown). Expression was sustained over a 72 hour period and the half life of the proteins



were at least 12 hours (work done by H. Bone and Charlotte Jago; results not shown). Figure V.3 shows typical expression levels of the FL (A), Y317F (B), SH2 (C), and PTB (D) Shc variants upon removal of tetracycline after approximately 24 hours. Transfectants were incubated at  $1 \times 10^5$  cells per ml and extracts were prepared 24 hours after removal of tetracycline and assayed for protein content, by Bradford assay, to ensure even loading of the gels. The proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with 9E10 antibodies to detect the myc tagged expressed Shc proteins (Fig. V.3 upper panels). The blots were stripped and reprobed with anti-Shc antibodies to compare the levels of expressed Shc variants to endogenous Shc (Fig. V.3 lower panels). FL Shc proteins were expressed 5 -20 fold that of endogenous p52<sup>Shc</sup> and Y317F and SH2 Shc proteins were expressed 10-50 fold that of endogenous p52<sup>Shc</sup>, as determined by densitometric analysis. However, the anti-Shc antibody was raised against the SH2 domain of Shc and hence will not detect the expressed PTB domain; therefore, comparison of the levels of expressed PTB protein to endogenous Shc cannot be determined using this antibody. The clones, shown in Fig. V.3, were used for subsequent analyses and are as follows: FL Shc 3E and 6G; Y317F Shc 8D and 8H; SH2 Shc 2A10 and 3C11; PTB Shc 4C and 4F.

#### **V.E . The expressed Shc variants interact with tyrosine phosphorylated proteins in response to IL-3.**

In order to determine if the expressed Shc proteins interact with a similar set of IL-3-induced tyrosine phosphorylated proteins as endogenous Shc and to confirm the interactions determined by *in vitro* analyses (see Chapter IV), precipitations using the anti-myc epitope antibody (9E10) from IL-3 stimulated transfectants expressing the various Shc constructs were performed. Tyrosine phosphorylated proteins which co-precipitate with Shc following treatment with IL-3 in murine cells have been identified *in vitro* using GST fusion proteins (see Chapter IV). These are proteins of 135-145 kDa, which represents the murine IL-3 R chain, Aic2A, as well as proteins of 130 and 140 which appear to be the inositol polyphosphate 5'-phosphatase, SHIP.

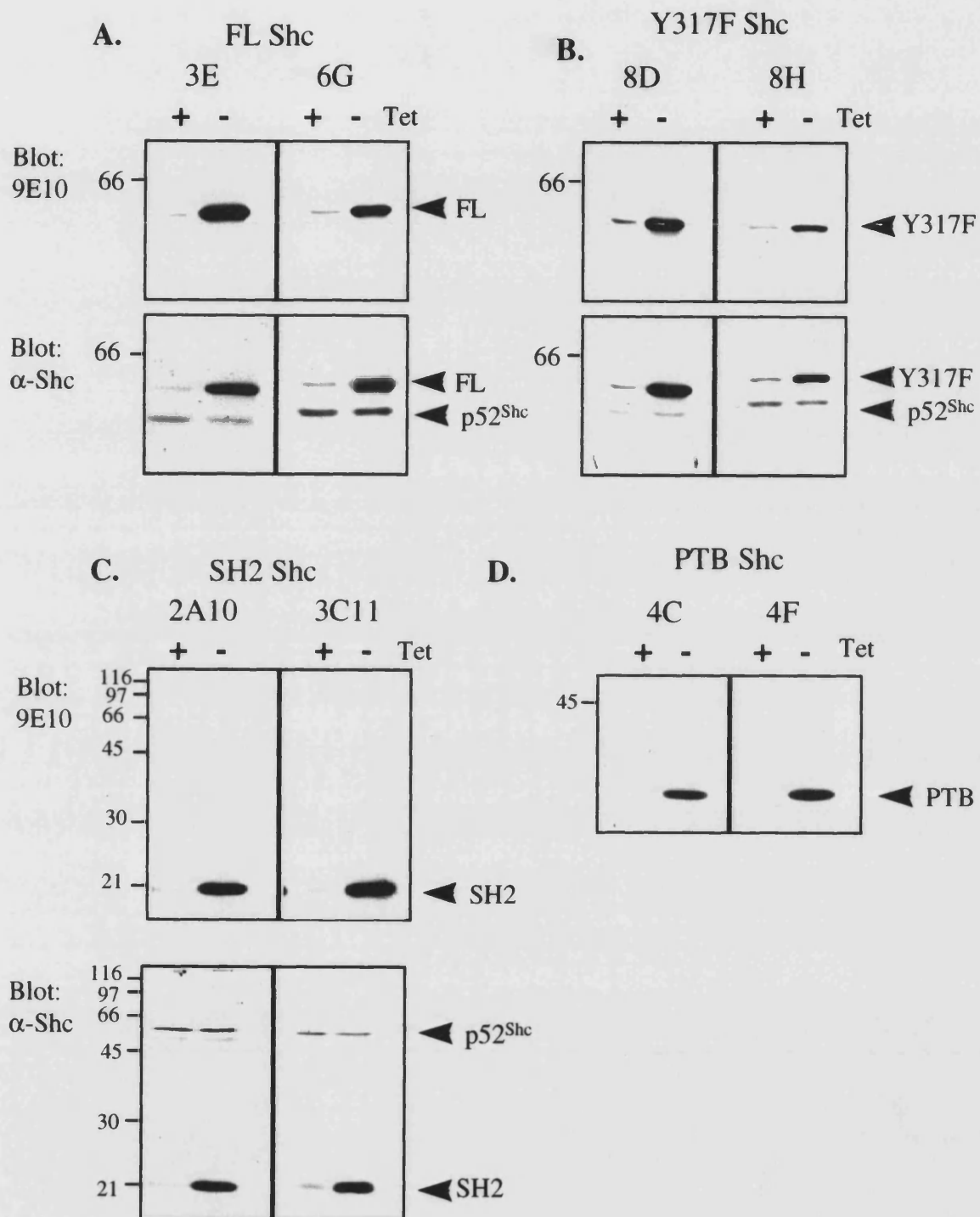
Cells were incubated at  $1 \times 10^5$  cells per ml in the presence of tetracycline or the absence of tetracycline for 16 hours to induce expression, and then either left untreated as a

### **Figure V.3**

#### **Expression of myc-tagged Shc proteins.**

Ba/F3 clones expressing FL Shc (**A**), the Y317F Shc mutant (**B**), the SH2 domain alone (**C**), or the PTB domain alone (**D**) were plated at  $1 \times 10^5$  cells per ml in the presence (+) or absence (-) of 2  $\mu\text{g/ml}$  tetracycline and cell extracts prepared after 24 hours. 15  $\mu\text{g}$  of protein was loaded in each lane and separated through 7.5% (**A,B**), 15% (**C**), or 12% (**D**) acrylamide gels by SDS-PAGE. The gels were transferred to nitrocellulose and immunoblotted with the 9E10 monoclonal antibody which recognises the myc tagged expressed Shc proteins (upper panels). The same immunoblots were then stripped and reprobed with anti-Shc antibodies (lower panels). Molecular mass standards are shown and expressed in kDa and the positions of the expressed and endogenous Shc proteins are indicated. The clones shown here, FL3E, FL 6G, Y317F 8D, Y317F 8H, SH2 2A10, SH2 3C11, PTB 4C and PTB 4F, were used for subsequent analyses.

**FIGURE V.3**



control (C) or stimulated with IL-3 (3) for 10 minutes. 9E10 immunoprecipitations were subsequently performed on the resulting cell extracts. The experiments were repeated twice and the results of one clone expressing each of the Shc variants are shown in Figure V.4, although similar results were observed with the other clone in each case. Anti-Shc immunoprecipitates from Ba/F3 cells (Fig. V.4 A) are shown for comparison. Anti-Shc antibodies precipitate the tyrosine phosphorylated proteins of 135-145 kDa and 130 and 140 kDa (which are Aic2A and SHIP (see section IV.E)) from Ba/F3 cells after IL-3 stimulation. (Fig. V.4 A). It appears that the FL (Fig. V.4 B), Y317F (Fig. V.4 C) and PTB (Fig. V.4 D) expressed Shc proteins are able to interact with similar tyrosine phosphorylated proteins. In addition, the expressed SH2 domain appears to be able to interact weakly with a tyrosine phosphorylated 135 kDa protein (Fig. V.4 E), likely to be Aic2A since the SH2 domain of Shc does not interact with SHIP (Liu *et al.*, 1994). It is interesting to note that the expressed Shc SH2 domain does not appear to interact with an IL-3-induced tyrosine phosphorylated protein of 100 kDa that was observed in ShcSH2-GST precipitations from IL-3-stimulated Ba/F3 cells (see section IV.F), suggesting that the interaction does not occur at detectable levels in this *in vivo* system. These results suggest that the expressed Shc proteins are capable of binding to a similar set of tyrosine phosphorylated proteins as endogenous Shc and confirm that the interactions of Shc with Aic2A and SHIP determined *in vitro* likely occur *in vivo*: the expressed FL, Y317F and PTB Shc proteins appear to be able to interact with both tyrosine phosphorylated Aic2A and SHIP whereas the expressed SH2 domain only binds one tyrosine phosphorylated protein of the size of Aic2A.

#### **V.F. Effects of expression of Shc variants on IL-3-induced tyrosine phosphorylation of cellular substrate.**

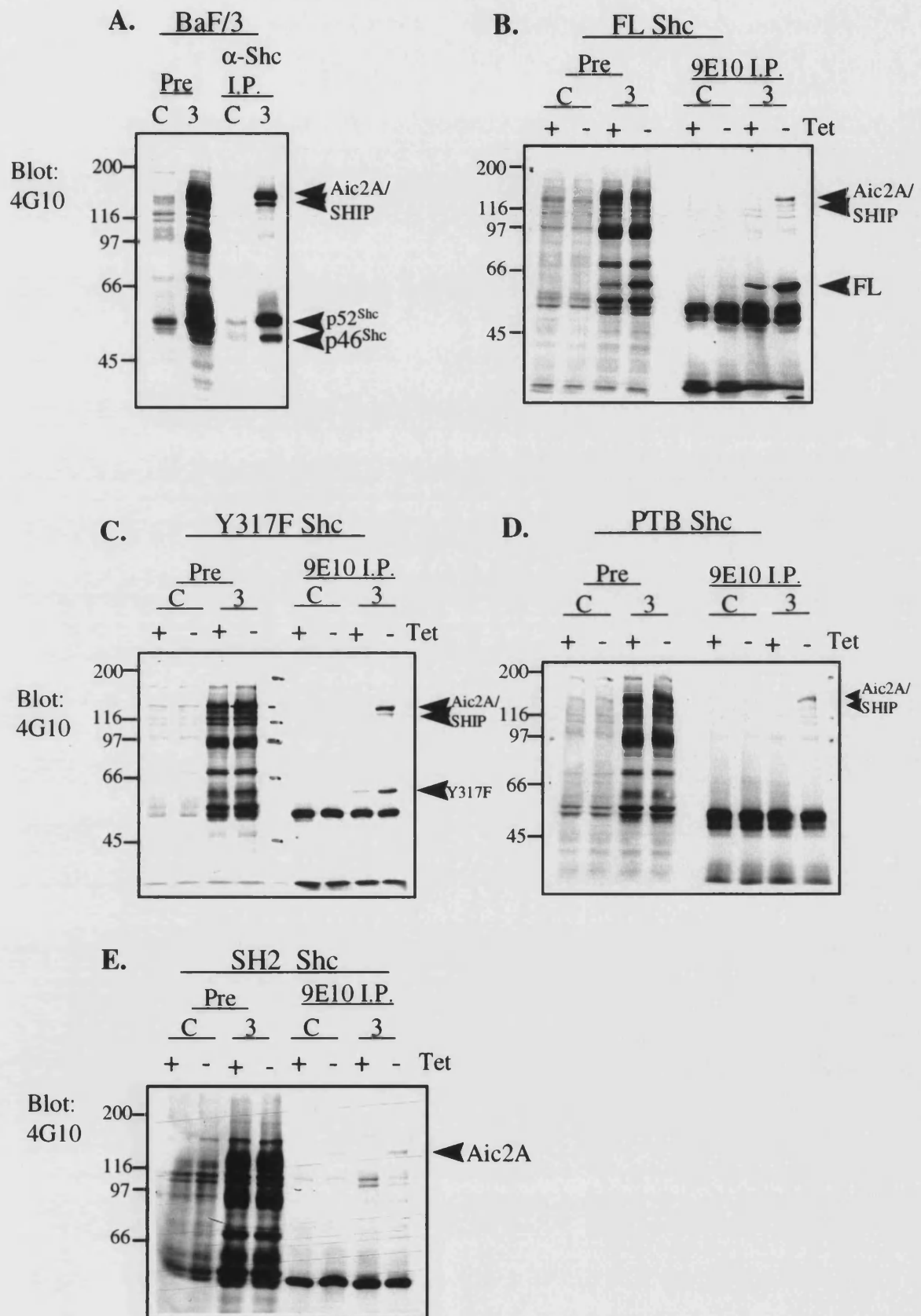
To determine if expression of any of the Shc variants has a dramatic effect on both the levels of IL-3-induced tyrosine phosphorylation and the substrates inducibly tyrosine phosphorylated in response to IL-3, the patterns of tyrosine phosphorylated cellular substrates induced by IL-3 was investigated in the various transfectants in both the presence and absence of induced expression. IL-3 time-course analyses, as well as specific immunoprecipitations, were performed.

#### **Figure V.4**

##### **Association of the expressed Shc variants with tyrosine phosphorylated proteins.**

(A) Ba/F3 cells were either left untreated as a control (C) or stimulated with 20 ng/ml rmIL-3 for 10 minutes (3). Cell extracts from the equivalent of  $1 \times 10^7$  cells were then precipitated with 2  $\mu$ g anti-Shc antibodies. Clones FL 3E (B), Y317F 8D (C), PTB 4F (D), and SH2 3C11 (E) were plated at  $1 \times 10^5$  cells per ml in the presence (+) or absence (-) of 2  $\mu$ g/ml tetracycline and incubated for 16 hours. Cells were washed and factor deprived for 45 minutes, then either left untreated as a control (C) or stimulated for 10 minutes with 20 ng/ml rmIL-3 (3). Cell extracts from the equivalent of  $1 \times 10^7$  cells were precipitated with 5 $\mu$ g 9E10 antibodies. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies. Molecular mass standards are shown in kDa and the positions of the 130 and 135 kDa proteins are indicated. These data presented in this figure are representative of two separate experiments with similar results observed for the other clone in each case.

**FIGURE V.4**

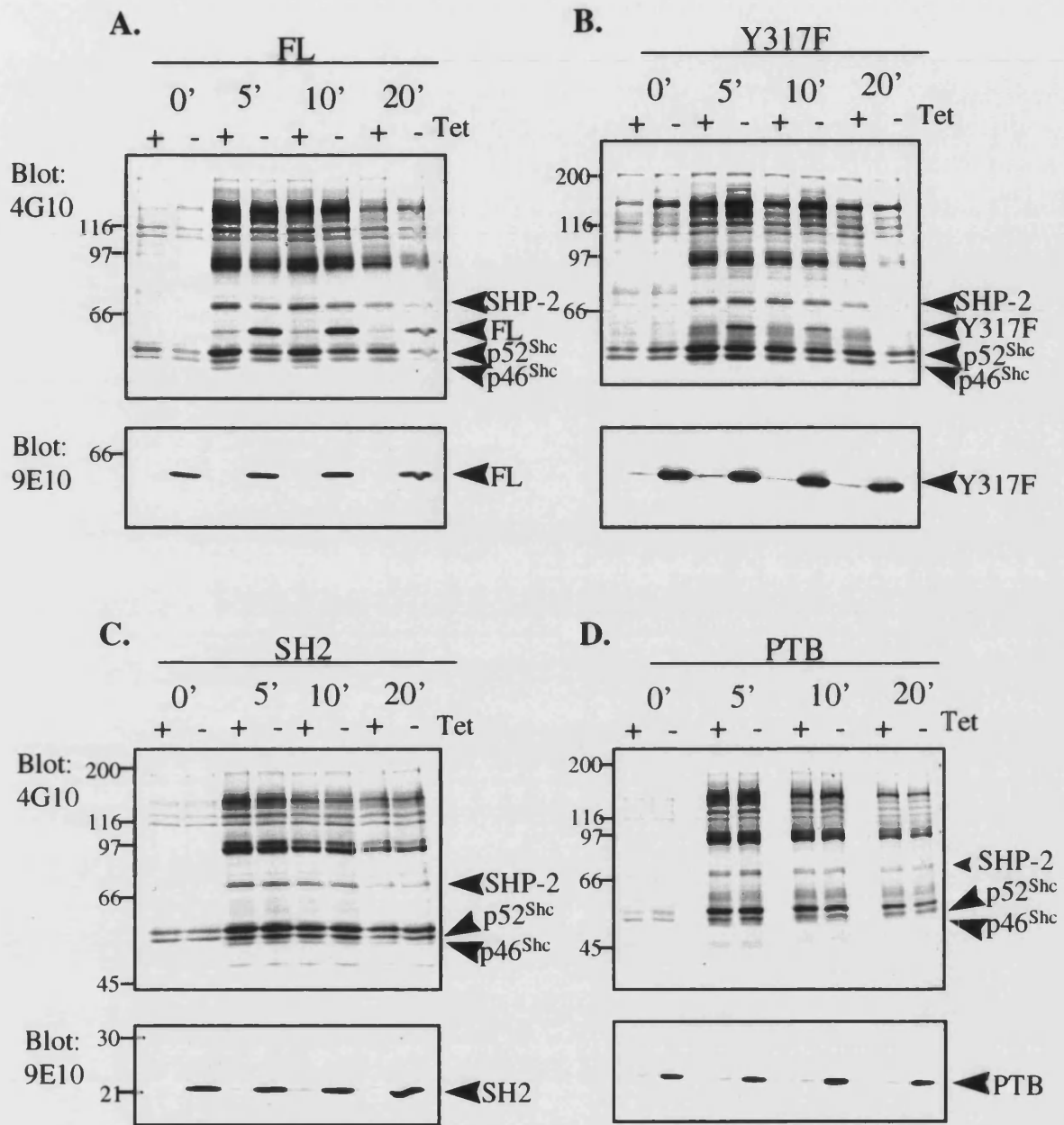


### V.F.1 Effects on overall IL-3-induced tyrosine phosphorylation.

IL-3 induces tyrosine phosphorylation of a number of cellular proteins including Aic2A, SHIP, p120<sup>JAK2</sup>, p90<sup>STAT5</sup>, p100, SHP-2, p46<sup>Shc</sup>, and p52<sup>Shc</sup> (see Introduction). In whole cell lysates from cells expressing the Shc proteins incubated in the presence or absence of tetracycline, IL-3 induces tyrosine phosphorylation of a number of proteins (Fig. V.5). As would be expected, in the presence of tetracycline (i.e.; no expression of Shc proteins), transfectants showed a very similar profile of tyrosine phosphorylated proteins as seen in normal Ba/F3 cells stimulated with IL-3 (see Fig. IV.3 B and IV.5 A). When FL Shc was expressed (Fig. V.5 A), there was a marked IL-3-induced tyrosine phosphorylation of the expressed FL Shc proteins but there also consistently appeared to be a reduction in IL-3-induced tyrosine phosphorylation of endogenous p52<sup>Shc</sup> and p46<sup>Shc</sup> which was observed in three separate experiments in both FL Shc expressing clones. A mean 2.9 fold reduction in endogenous p52<sup>Shc</sup> phosphorylation in cells overexpressing FL Shc was determined by densitometry and statistical analysis (presented in Appendix I;  $p=0.1153$ ). In addition, there also appeared to be a slight decrease in IL-3-induced tyrosine phosphorylation of SHP-2, but this was not observed consistently. Expression of the FL Shc protein was greater than 10 fold that of endogenous p52<sup>Shc</sup> (as determined by densitometer readings of an anti-Shc re-probe; results not shown).

In cells expressing the Y317F mutant Shc protein (Fig. V.5 B), the Y317F Shc protein was only moderately tyrosine phosphorylated in response to IL-3 owing to the fact that the major site of phosphorylation, tyrosine 317, was mutated to phenylalanine. However, this results implies that there are other tyrosine(s) within Shc capable of being phosphorylated in response to IL-3. An apparent slight decrease in IL-3-induced endogenous Shc phosphorylation was observed in clones expressing the Y317F mutant Shc protein. The time-course analyses were performed three times and similar results were observed for both clones with the levels of Y317F Shc expression 10 fold that of endogenous p52<sup>Shc</sup> (as determined by densitometer readings; results not shown). A mean 1.8 fold reduction in endogenous p52<sup>Shc</sup> phosphorylation in cells overexpressing Y317F Shc was determined by densitometry and statistical analysis (presented in Appendix I;  $p=0.1223$ ). Decreases in endogenous Shc phosphorylation were also consistently observed

**FIGURE V.5**



**Effects on IL-3 induced tyrosine phosphorylation in whole cell extracts from cells expressing Shc variants.**

Clones FL 3E (A), Y317F 8D (B), SH2 3C11 (C), and PTB 4F (D) were plated at  $1 \times 10^5$  per ml in the presence (+) or absence (-) of 2  $\mu$ g/ml tetracycline for 16 hours. Cells were then factor deprived for 45 minutes before being stimulated with 20 ng/ml rmIL-3 for the indicated times. Samples of total cell extracts were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 antibodies (upper panels). The blots in A and B were stripped and reprobed with 9E10 antibodies to detect the expressed proteins (lower panels). Samples from the same whole cell extracts were separated on separate 12% (C lower panel) and 10% (D lower panel) gels and immunoblotted with 9E10 antibodies. The molecular mass standards are shown and expressed in kDa and the positions of the expressed proteins, p52<sup>Shc</sup>, p46<sup>Shc</sup>, and SHP-2 are indicated. The data presented in this figure are representative of 2-3 separate experiments with similar results observed for the other clone in each case.



in FL and Y317F Shc expressing cells in pre-immunoprecipitation extracts from other experiments.

Expression of the SH2 domain (Fig. V.5 C) or PTB domain (Fig. V.5 D) alone appeared to have no detectable effect on IL-3-induced tyrosine phosphorylation of cellular substrates over a period of 20 minutes, even though the SH2 Shc protein was expressed up to 50 fold that of endogenous p52<sup>Shc</sup>. All time-course analyses were performed at least twice and similar results were also observed in whole cell extracts from other experiments. Therefore, the only consistent detectable effects seen on IL-3-induced tyrosine phosphorylation of protein substrates from whole cell extracts of cells expressing the various Shc mutants appeared to be a moderate reduction in endogenous Shc phosphorylation in cells expressing FL Shc and a slight reduction in endogenous Shc phosphorylation in cells expressing the Y317F mutant Shc.

#### **V.F.2 Effects of expression of Shc variants on endogenous Shc phosphorylation.**

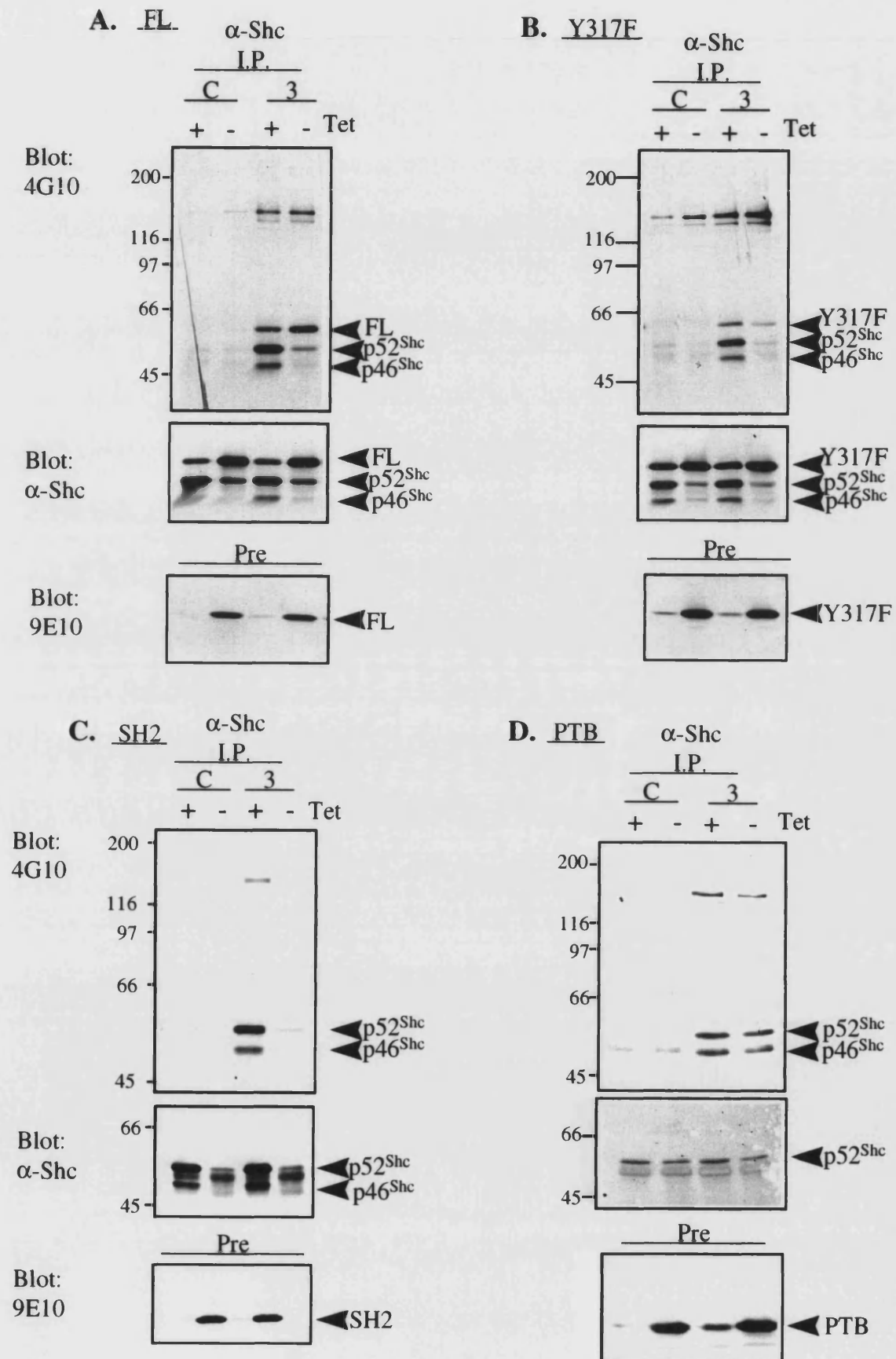
Having observed a slight reduction in IL-3-induced endogenous Shc phosphorylation in cells expressing FL Shc and Y317F Shc, this was investigated further. Assuming that Shc is required to bind to the IL-3 receptor in order to become tyrosine phosphorylated, expression of these Shc mutants may inhibit phosphorylation of endogenous Shc by competing with endogenous Shc for the binding to activated Aic2A. Anti-Shc immunoprecipitates were performed and then immunoblotted with 4G10 anti-phosphotyrosine antibodies to specifically observe the effects of expression of the Shc variants on endogenous Shc phosphorylation (Fig. V.6 upper panels). As was seen in the whole cell lysates, there appeared to be a reduction in IL-3-induced endogenous Shc phosphorylation in cells expressing FL (Fig. V.6 A) and Y317F (Fig. V.6 B) but not in cells expressing the PTB domain alone (Fig. V.6 D). However, in these anti-Shc immunoprecipitates, there also appeared to be a pronounced reduction in endogenous Shc phosphorylation in cells expressing the SH2 domain (Fig. V.6 C). Gotoh *et al.* (1996) had also observed a similar reduction in endogenous tyrosine phosphorylated Shc in Ba/F3 cells expressing the SH2 domain in an inducible manner. However, when the blots in Figure

### Figure V.6

#### IL-3-induced endogenous Shc phosphorylation in cells expressing Shc variants.

Clones FL 3E (A), Y317F 8D (B), SH2 2A10 (C), and PTB 4C (D) were plated at  $1 \times 10^5$  per ml in the presence (+) or absence (-) of 2  $\mu\text{g/ml}$  tetracycline for 16 hours. Factor deprived cells were then either left untreated as a control (C) or stimulated with 20 ng/ml rmIL-3 for 10 minutes (3). Cell extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with 2  $\mu\text{g}$  anti-Shc antibodies. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 antibodies (upper panels). The blots were then stripped and reprobed with anti-Shc antibodies (middle panels). To detect the expressed proteins, samples of the total cell extracts were removed prior to precipitation and separated through 7.5% (A and B lower panels), 15% (C) or 10% (D) acrylamide gels and immunoblotted with 9E10 antibodies. Molecular mass standards are shown in kDa and the positions of the expressed proteins, p52<sup>Shc</sup>, and p46<sup>Shc</sup> are indicated. These data presented in this figure are representative of two-four separate experiments with similar results observed for the other clone in each case.

**FIGURE V.6**



V.6 were stripped and reprobed with anti-Shc antibodies (middle panel), an interesting phenomenon was observed. In cells expressing FL (A), Y317F (B), or the SH2 domain alone (C), there was a marked reduction in the amount of endogenous Shc being precipitated from the cells in both unstimulated and IL-3-stimulated samples. Therefore, it appeared that the expressed protein was competing with endogenous Shc for a limited pool of anti-Shc antibodies resulting in less endogenous Shc being precipitated from the cell. This was not observed in cells expressing the PTB domain alone as the antibody is directed towards the SH2 domain of Shc and therefore cannot bind to the PTB domain alone. Gotoh *et al.* (1996) failed to perform this extra re-probing step and therefore the reduction they saw in endogenous Shc phosphorylation resulting in expression of the SH2 domain was most likely due to this phenomenon and not due to the SH2 domain specifically and competitively inhibiting tyrosine phosphorylation of endogenous Shc.

To overcome the anti-Shc antibody problem, Grb2SH2-GST precipitations were performed on cells expressing FL, Y317F and the Shc SH2 domain (Fig. V.7). The Grb2SH2-GST fusion protein should only precipitate Shc tyrosine phosphorylated at Y317 and therefore not bind significantly to the expressed Y317F Shc mutant or to the Shc SH2 domain alone. Additionally, at 10  $\mu\text{g/ml}$  the Grb2SH2-GST fusion protein should not be limiting so even though it can bind to the expressed FL Shc protein there ought to be excess remaining to precipitate endogenous Shc. In cells expressing FL Shc (Fig. V.7 A), there was a reduction in IL-3-induced tyrosine phosphorylated endogenous  $\text{p52}^{\text{Shc}}$  and  $\text{p46}^{\text{Shc}}$  precipitated by the Grb2SH2-GST fusion protein. Densitometric analysis of three individual experiments indicated a mean 2.4 fold decrease in endogenous  $\text{p52}^{\text{Shc}}$  phosphorylation in cells expressing FL Shc (statistical analysis presented in Appendix I;  $p=0.2012$ ). The Grb2 SH2 domain can also interact with tyrosine phosphorylated SHP-2 (Welham *et al.*, 1994b). Expression of FL Shc did not appear to affect the amount of IL-3-induced tyrosine phosphorylated SHP-2 precipitated by the Grb2 SH2 domain. In cells expressing the Y317F mutant, there was also a reduction in IL-3-induced tyrosine phosphorylated endogenous  $\text{p52}^{\text{Shc}}$  and  $\text{p46}^{\text{Shc}}$  precipitated by the Grb2SH2-GST fusion protein (Fig. V.7 B). A mean 2.2 fold decrease in endogenous  $\text{p52}^{\text{Shc}}$  phosphorylation in cells expressing Y317F Shc was determined by densitometric analysis of three separate experiments (statistical analysis presented in Appendix I;  $p=0.1347$ ). There was a small

### **Figure V.7**

#### **IL-3-induced endogenous Shc phosphorylation is decreased in cells expressing FL and Y317F Shc but not the SH2 domain alone.**

Clones FL 3E (A), Y317F 8D (B), and SH2 3C11 (C) were plated at  $1 \times 10^5$  per ml in the presence (+) or absence (-) of 2  $\mu\text{g/ml}$  tetracycline for 16 hours. Factor deprived cells were then either left untreated as a control (C) or stimulated with 20 ng/ml rmIL-3 for 10 minutes (3). Cell extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with 10  $\mu\text{g}$  Grb2SH2-GST fusion protein. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 antibodies (upper panels). To detect the expressed proteins, samples of the total cell extracts were removed prior to precipitation and separated through 7.5% (A and B lower panels), or 12% (C lower panel) acrylamide gels and immunoblotted with 9E10 antibodies. The positions of the molecular mass standards are shown and expressed in kDa and the positions of the expressed proteins, p52<sup>Shc</sup>, p46<sup>Shc</sup>, and SHP-2 are indicated. These data presented here are representative of two-five separate experiments with similar results observed for the other clone in each case.

**FIGURE V.7**



amount of tyrosine phosphorylated expressed Y317F Shc protein precipitated by Grb2SH2-GST, suggesting that other tyrosine phosphorylated site(s) on Shc could interact with the SH2 domain of Grb2 *in vitro*. Again, there was no reduction in tyrosine phosphorylated SHP-2 precipitated by Grb2SH2-GST fusion protein in cells expressing the Y317F mutant. However, expression of the Shc SH2 domain alone (Fig. V.7 C) had no effect on endogenous IL-3-induced tyrosine phosphorylated p52<sup>Shc</sup> and p46<sup>Shc</sup> precipitated by Grb2SH2-GST, contrary to what was initially observed in the anti-Shc immunoprecipitates (Fig. V.6 C). Again, no effect on IL-3-induced tyrosine phosphorylated SHP-2 precipitated by GrbSH2-GST was observed in cells expressing the SH2 domain alone. Therefore, it appears that expression of the FL and Y317F mutant Shc proteins caused a reduction in IL-3-induced endogenous Shc tyrosine phosphorylation whereas expression of the SH2 or PTB domain alone had no effect on IL-3-induced Shc tyrosine phosphorylation.

### V.F.3 Effects of expression of Shc variants on SHP-2 tyrosine phosphorylation.

A slight reduction in IL-3-induced SHP-2 tyrosine phosphorylation was observed in whole cell lysates from cells expressing FL Shc (Fig. V.5 A) but a similar reduction was not seen in Grb2SH2-GST precipitates (Fig V.7 A) (or in cells expressing Y317F or SH2). In human cells, SHP-2 appears to bind to residues surrounding the same  $\beta$ c phosphotyrosine (Y 612) as the SH2 domain of Shc. Assuming SHP-2 is required to bind to Aic2A in order to become phosphorylated, it is possible that the expressed Shc variants could compete with SHP-2 for binding to Aic2A and hence affect SHP-2 phosphorylation. To examine total cellular IL-3-induced SHP-2 tyrosine phosphorylation (instead of only that associated with the SH2 domain of Grb2), anti-SHP-2 immunoprecipitates were performed on cells expressing the Shc variants (Figure V.8). There did not appear to be a significant effect on IL-3-induced SHP-2 tyrosine phosphorylation upon overexpression of FL (A), Y317F (B), SH2 (C) or PTB (D) Shc variants. Expression of the Shc variants also had no significant effect on tyrosine phosphorylation of the 100 kDa protein co-precipitating with SHP-2. The 130 kDa protein precipitated in the anti-SHP-2 immunoprecipitates is most likely Aic2A since it has been shown that SHP-2 can bind directly via its SH2 domain to  $\beta$ c in TF-1 cells (section III.C). The precipitation of tyrosine

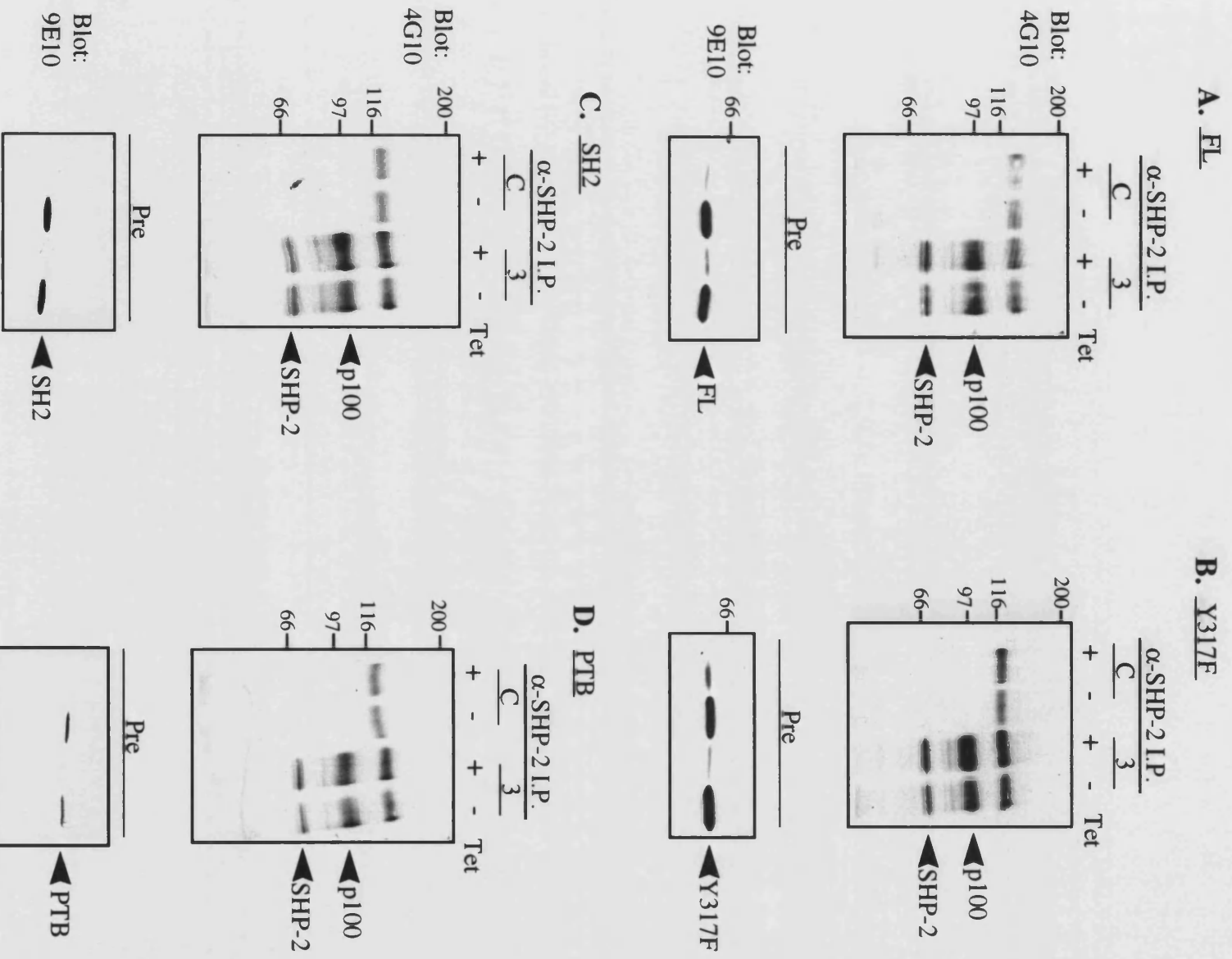
### **Figure V.8**

#### **IL-3-induced SHP-2 phosphorylation is not significantly affected in cells expressing Shc variants.**

Clones FL 3E (A), Y317F 8D (B), SH2 3C11 (C), and PTB 4F (D) were plated at  $1 \times 10^5$  per ml in the presence (+) or absence (-) of 2  $\mu\text{g/ml}$  tetracycline for 16 hours. Factor deprived cells were then either left untreated as a control (C) or stimulated with 20 ng/ml rmIL-3 for 10 minutes (3). Cell extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with 0.4  $\mu\text{g}$  anti-SHP-2 antibodies. All samples were separated through 7.5% acrylamide gels by SDS-PAGE and immunoblotted with 4G10 antibodies (upper panels). The blots in A and B were stripped and reprobbed with 9E10 antibodies to detect the expressed proteins (lower panels). Samples from the same whole cell extracts were separated on separate 12% gels (C and D lower panels) and immunoblotted with 9E10 antibodies. The molecular mass standards are shown and expressed in kDa and the positions of the expressed proteins, SHP-2 and p100 are indicated. These data depicted in this figure are representative of 2 separate experiments with similar results observed for the other clone in each case.



**FIGURE V.8**



phosphorylated Aic2A from the unstimulated cells probably reflects inadequate starvation of the cells prior to preparation of the cell extracts.

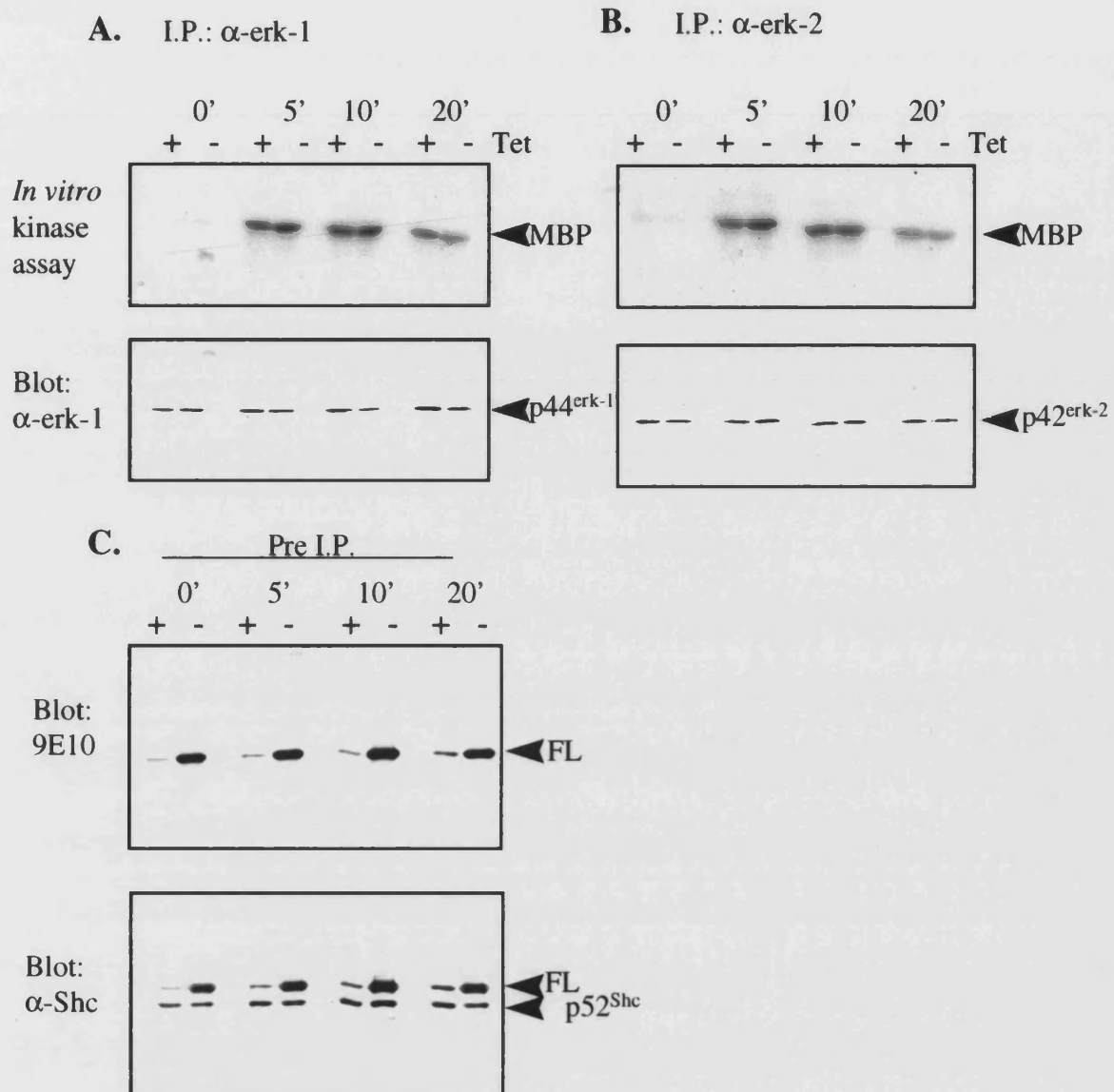
### **V.G. Effects of expression of Shc variants on IL-3-induced MAP kinase activation.**

IL-3 has been shown to induce activation of ras, erk1 and erk2 (Duronio *et al.*, 1992b; Welham *et al.*, 1992) and Shc has been implicated as an adaptor molecule involved in controlling protein-protein interactions important in the activation of the Ras/MAP kinase pathway by its ability to associate with the Grb2 adaptor molecule (Rozakis-Adcock *et al.*, 1992). Phosphorylation of Shc at tyrosine 317 provides a high affinity binding site for the SH2 domain of Grb2 (Salcini *et al.*, 1994). Given the ability of Shc to bind to the activated the IL-3 receptor via its PTB and SH2 domains *in vitro* (see section IV.E), this could provide a means of localising the Grb2-Sos complex to the plasma membrane where activation of Ras could occur and subsequently activate the MAP kinases erk1 and erk2. Therefore, expression of the Y317F Shc mutant or the SH2 or PTB domains may have an inhibitory affect on IL-3-induced activation of the MAP kinases erk1 and erk2. To determine the functional importance of Shc in regulating IL-3-induced activation of these MAP kinases, the ability of the cells expressing the Shc variants to activate erk1 and erk2 in response to IL-3 was examined.

#### **V.G.1 Expression of FL Shc.**

To examine the effects of expression of FL Shc on IL-3 activation of the erk1 and erk2 members of the MAP kinase family, *in vitro* kinase assays were first performed (Fig. V.9). Cells were incubated at  $1 \times 10^5$  cells per ml with or without 2  $\mu\text{g/ml}$  tetracycline for 16 hours then stimulated with 20 ng/ml IL-3 (concentration required for maximum tyrosine phosphorylation of cellular substrates) for 5, 10, or 20 minutes or left untreated as a control. Erk1 and erk2 kinases were immunoprecipitated from cell extracts from the equivalent of  $5 \times 10^6$  cells and *in vitro* kinase assays performed on immunoprecipitations with MBP as an exogenous substrate (see section II.C.10). Assays were performed twice and similar results were obtained for both FL clones. There appeared to be no significant effect on IL-3-induced erk1 (Fig. V.9 A, upper panel) or erk2 (Fig. V.9 B, upper panel)

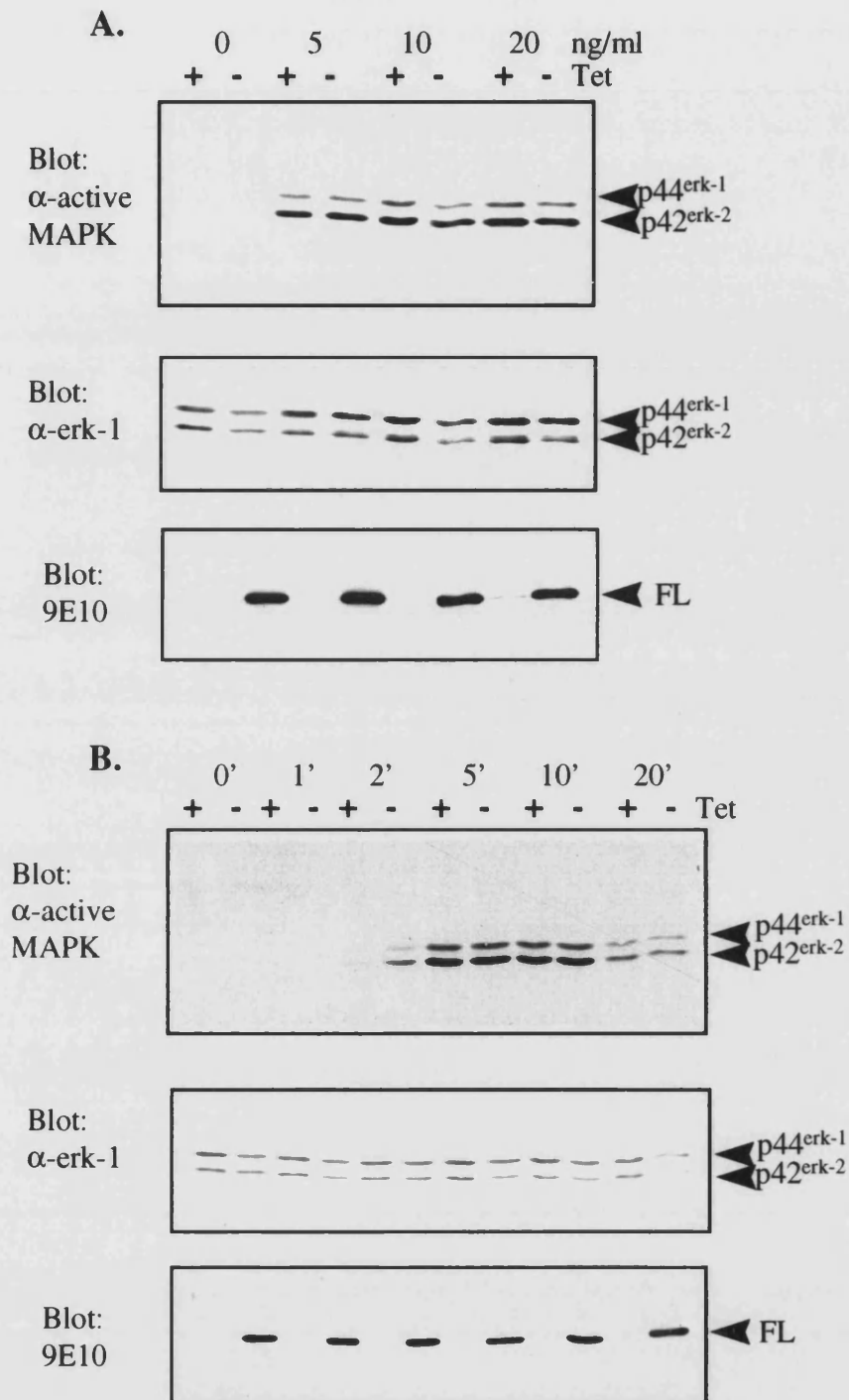
**FIGURE V.9**



**Expression of FL Shc does not have a significant effect on IL-3 induced MAP kinase activation in *in vitro* kinase assays.**

Clone FL 6G was incubated at  $1 \times 10^5$  cell per ml in the presence (+) or absence (-) of 2  $\mu$ g/ml tetracycline for 16 hours. Factor deprived cells were then either left untreated as a control or stimulated with 20 ng/ml rmIL-3 for the indicated times. Cell extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with either anti-erk1 (A) or anti-erk2 (B) agarose conjugated beads and *in vitro* kinase assays were performed in the presence of MBP. The samples were separated on a 15% acrylamide gel by SDS-PAGE and transferred to nitrocellulose. The bottom half of the blot was subjected to autoradiography and the incorporation of  $^{32}$ P into MBP is indicated (A and B upper panels). Immunoblotting the top half of the blot with anti-MAP kinase antibodies (A and B lower panels) confirms equal precipitation of erk1 and erk2 (indicated). Samples of the total cell extracts were removed prior to precipitation and separated through a 7.5% acrylamide gel and immunoblotted with 9E10 antibodies for detection of the expressed FL Shc protein (C upper panel). The blot was then stripped and reprobed with anti-Shc antibodies (C lower panel). The positions of the expressed protein and p52<sup>Shc</sup> are indicated. Similar results were obtained with clone FL 3E.

**FIGURE V.10**



**Activation of erk1 and erk2 in response to IL-3 is moderately accelerated in FL Shc expressing cells.**

Clone FL 3E was plated at  $1 \times 10^5$  cells per ml in the presence (+) or absence (-) of 2  $\mu$ g/ml tetracycline for 16 hours. (A) Cells were treated for 5 minutes with the indicated concentration of rmIL-3 or (B) stimulated with 5 ng/ml rmIL-3 for the indicated periods of time. Samples were separated through 10% acrylamide gels and immunoblotted with an antibody specific for the activated forms of p44<sup>erk1</sup> and p42<sup>erk2</sup> MAP kinases (upper panels). The same blots were stripped and reprobed with anti-MAP kinase antibodies to demonstrate equal precipitation of erk1 and erk2 (middle panels) and further reprobed with 9E10 antibodies for detection of the expressed protein (lower panels). The positions of the expressed FL Shc protein, erk1 and erk2 are indicated. These data presented in this figure are representative of two separate time-course and dose-response experiments.

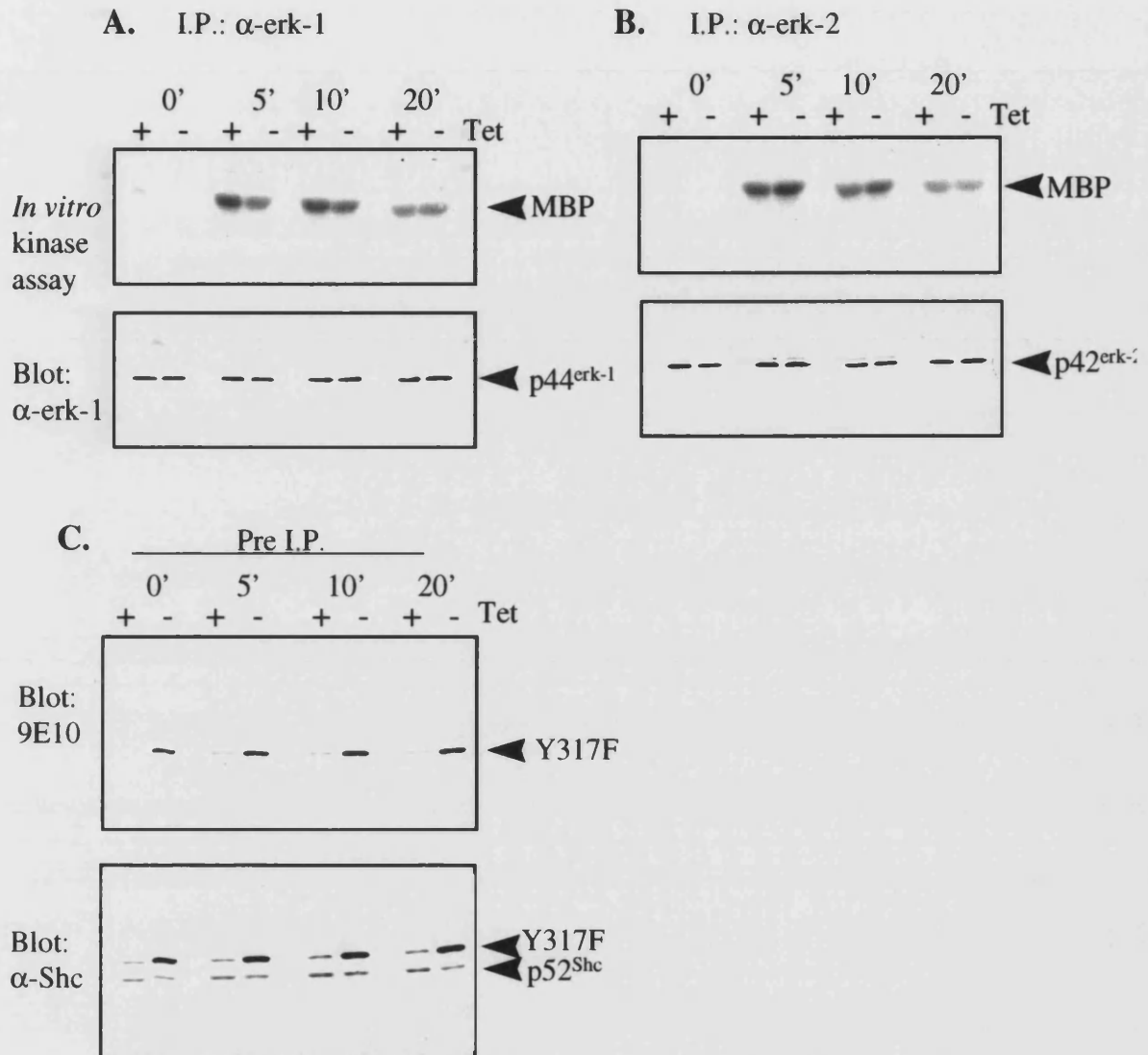
activation upon expression of FL Shc, even though there was significant expression of FL Shc (Fig. V.9 C) which was determined to be 15 fold that of endogenous p52<sup>Shc</sup> (determined by densitometer readings). Equal amounts of erk1 (Fig. V.9 A, lower panel) and erk2 (Fig. V.9 B, lower panel) were precipitated in each case.

IL-3 dose-response and time-course analyses and subsequent immunoblotting with anti-active MAP kinase antibodies were next performed to investigate in more detail if expression of the FL Shc protein was having any effects at sub-maximal concentrations of IL-3 or on the kinetics of MAP kinase activation. Again, cells were set up at  $1 \times 10^5$  cells per ml for 16 hours with or without 2  $\mu$ g/ml tetracycline and then either stimulated for 5 minutes with varying concentrations of IL-3 (Fig V.10 A) or stimulated with 5 ng/ml IL-3 for varying times (Fig. V.10 B). Cell extracts were immunoblotted with antibodies specific to threonine and tyrosine phosphorylated erk1 and erk2 to specifically detect activated forms of the two enzymes. No significant change in erk1 or erk2 activation was observed over an IL-3 dose-response at the 5 minute time point when FL Shc was expressed (Fig. V.10 A), even though there was good inducible expression (Fig. V.10 A, lower panel). However, expression of FL Shc appeared to cause a slight increase in erk1 and erk2 activation after stimulation with 5 ng/ml rmIL-3 for 2 minutes, which was equivalent to cells not expressing FL Shc after 5 minutes. (Fig. V.10 B, upper panel). These results were observed in two separate experiments. This may suggest a slight enhancement in the rate of activation of erk1 and erk2 upon expression of FL Shc. However, expression of FL Shc did not detectably effect the maximal level of erk1 and erk2 MAP kinase activation.

### V.G.2 Expression of Y317F Shc.

To examine the effects on IL-3 activation of erk1 and erk2 upon expression of the Y317F Shc mutant, *in vitro* kinase assays were first performed. Similar results were obtained for both Y317F Shc clones and the results from one clone are shown in Figure V.11. *In vitro* kinase assays were performed as for the FL expressing clones (see section V.G.1). At 20 ng/ml, over times ranging up to 20 minutes, no significant effect on IL-3-induced activation of the erk1 (Fig. V.11 A) and erk2 (Fig. V.11 B) kinases were observed when the mutant Y317F Shc was expressed, although there were high levels of expressed protein (Fig. V.11 C) which was determined to be 10 fold that of endogenous p52<sup>Shc</sup> (as

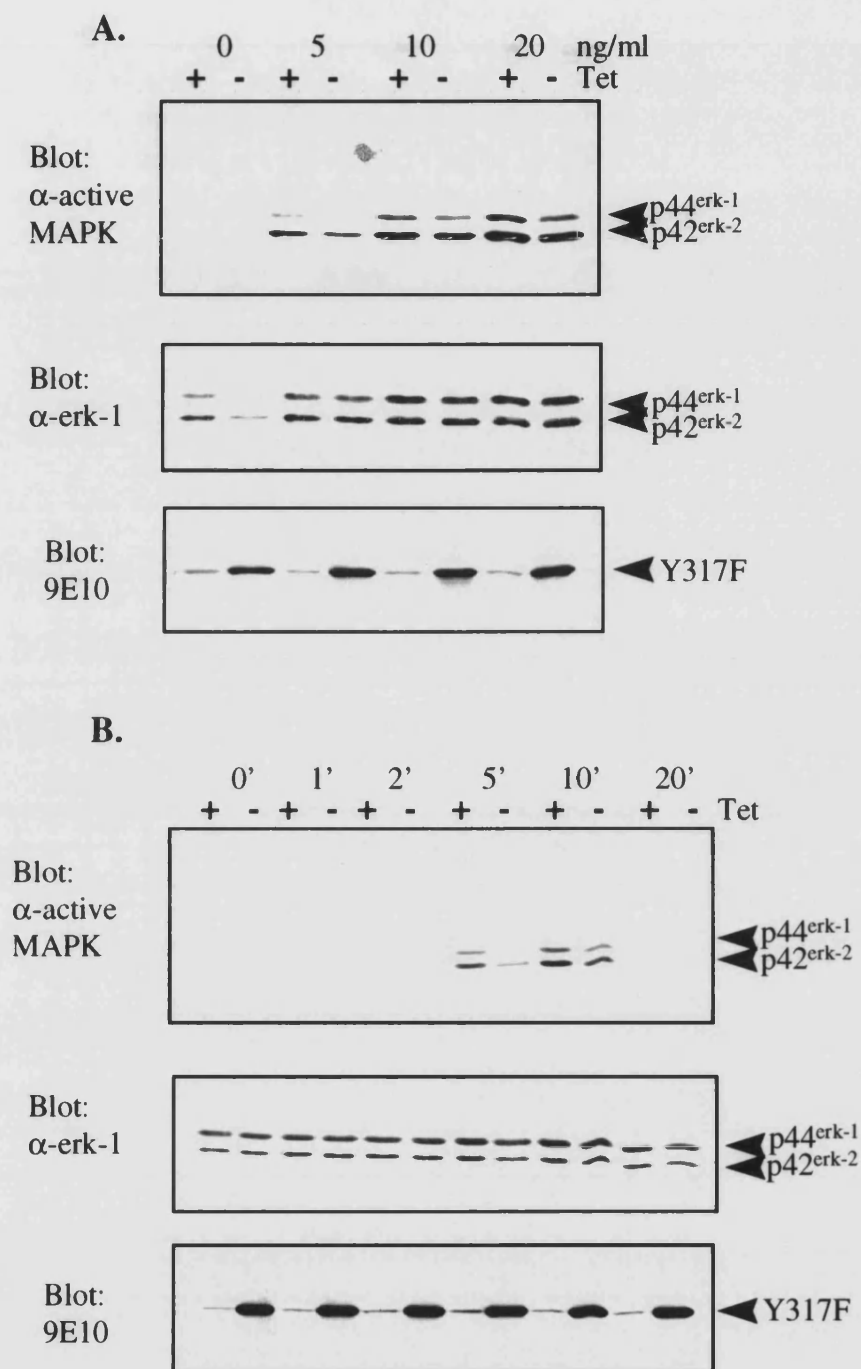
**FIGURE V.11**



**Expression of Y317F Shc does not have a significant effect on IL-3 induced MAP kinase activation observed in *in vitro* kinase assays.**

Clone Y317F 8H was plated at  $1 \times 10^5$  cells per ml in the presence (+) or absence (-) of 2  $\mu$ g/ml tetracycline for 16 hours. Erk1 (**A**) and erk2 (**B**) *in vitro* kinase assays were performed as described in the legend to Fig. V.9. **A** and **B** upper panels show incorporation of  $^{32}$ P into MBP. Immunoblotting with anti-MAP kinase antibodies confirms equal loading (**A** and **B** lower panels). Samples of the total cell extracts prior to precipitation were separated through a 7.5% gel and immunoblotted with 9E10 antibodies for detection of the expressed protein (**C** upper panel), then stripped and reprobed with anti-Shc antibodies (**C** lower panel). The positions of erk1, erk2, p52<sup>Shc</sup> and the expressed Y317F Shc protein are indicated. Similar results were obtained with clone Y317F 8D.

**FIGURE V.12**



**Activation of erk1 and erk2 in response to IL-3 is decreased in Y317F Shc expressing cells.**

(A) IL-3 dose response and (B) time course analyses were performed on clone Y317F 8D as described in the legend to Figure V.10. The positions of the expressed Y317F Shc protein, erk1 and erk2 are indicated. The data depicted here is representative of two separate time-course and dose-response experiments.

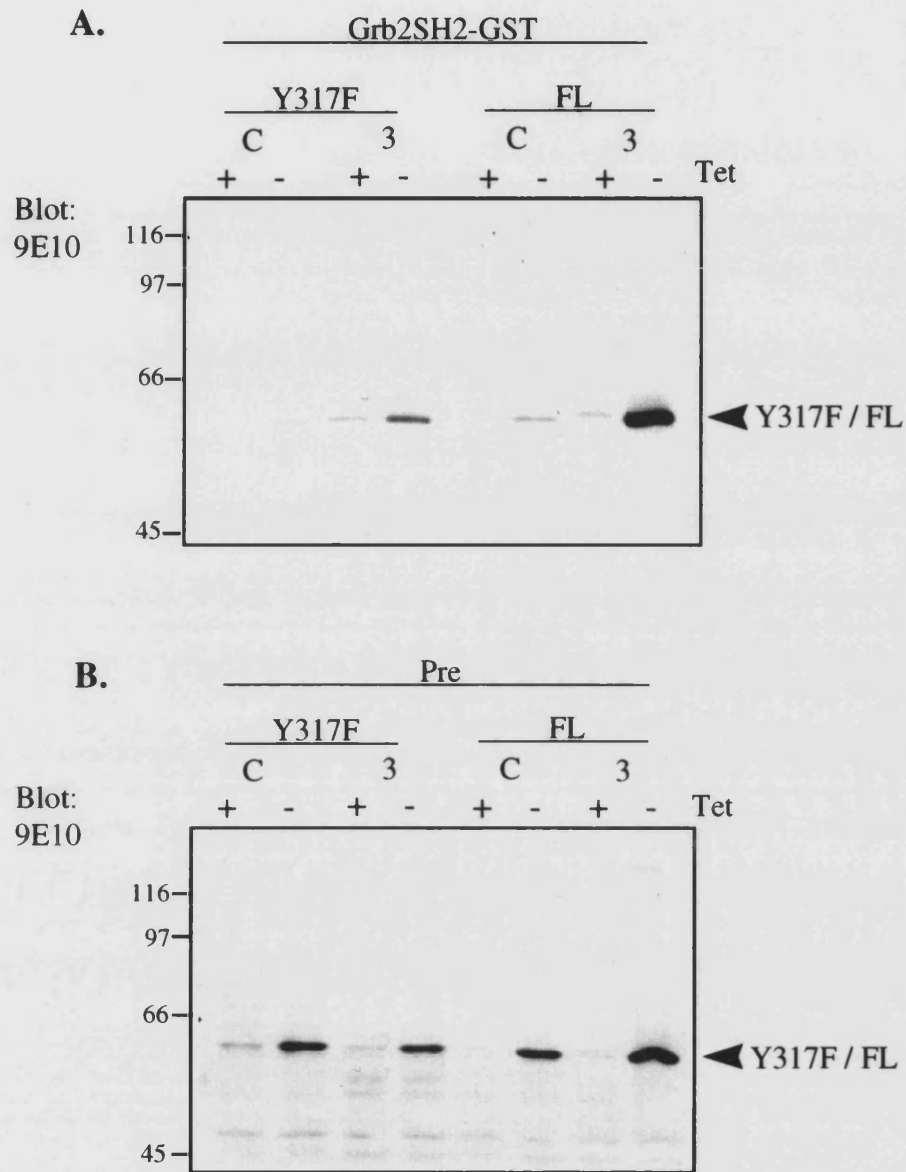
determined by densitometer readings). Equal amounts of erk1 (A, lower panel) and erk2 (B, lower panel) were precipitated in each experiment. However, more detailed dose-response and time-course analyses and immunoblotting with the anti-active MAP kinases antibody revealed an apparent modest decrease in IL-3-induced MAP kinase activation upon expression of the Y317F mutant (this was observed in two separate time-course and dose-response experiments) (Fig. V.12). IL-3 dose-response analyses (Fig. V.12 A) revealed a slight decrease in erk1 and erk2 activation after stimulation with 5 and 10 ng/ml IL-3 for 5 minutes when the Y317F mutant Shc protein was expressed. Only a very slight decrease was observed in Y317F expressing cells stimulated with 20 ng/ml rmIL-3 (this was the concentration used in the *in vitro* kinase assay). Re-probing the same blot with anti-erk antibodies revealed that the gel was evenly loaded (middle panel) and the 9E10 immunoblot shows good inducible expression of the Y317F protein (lower panel). A consistent decrease in IL-3-stimulated erk1 and erk2 activation was also observed in the time-course analysis (Fig. V.12 B) after stimulation with 5 ng/ml IL-3 for 5 and 10 minutes in cells expressing Y317F Shc. Re-probing the same blot with anti-erk antibodies demonstrated equal loading (middle panel) and again there was good inducible expression of the Y317F Shc protein (lower panel).

### **V.G.3. The expressed Y317F Shc mutant does not interact well with the SH2 domain of Grb2.**

Since it has been thought that the major role for Shc is in activation of the Ras/MAP kinase pathway, by the virtue of the SH2 domain of Grb2 binding Y317 of Shc, it was somewhat surprising that a more pronounced reduction in IL-3-induced MAP kinase activation was not observed in cells expressing the Y317F Shc mutant. One of the reasons could be because the mutant was able to interact with the SH2 domain of Grb2 via different phosphotyrosine(s). To investigate this, Grb2SH2-GST precipitations were performed on cells expressing FL Shc or the mutant Y317F Shc and immunoblotted with 9E10 to detect the precipitated expressed proteins (Fig. V.13 A).



**FIGURE V.13**



**Interaction of the expressed FL and Y317F Shc proteins with the SH2 domain of Grb2.**

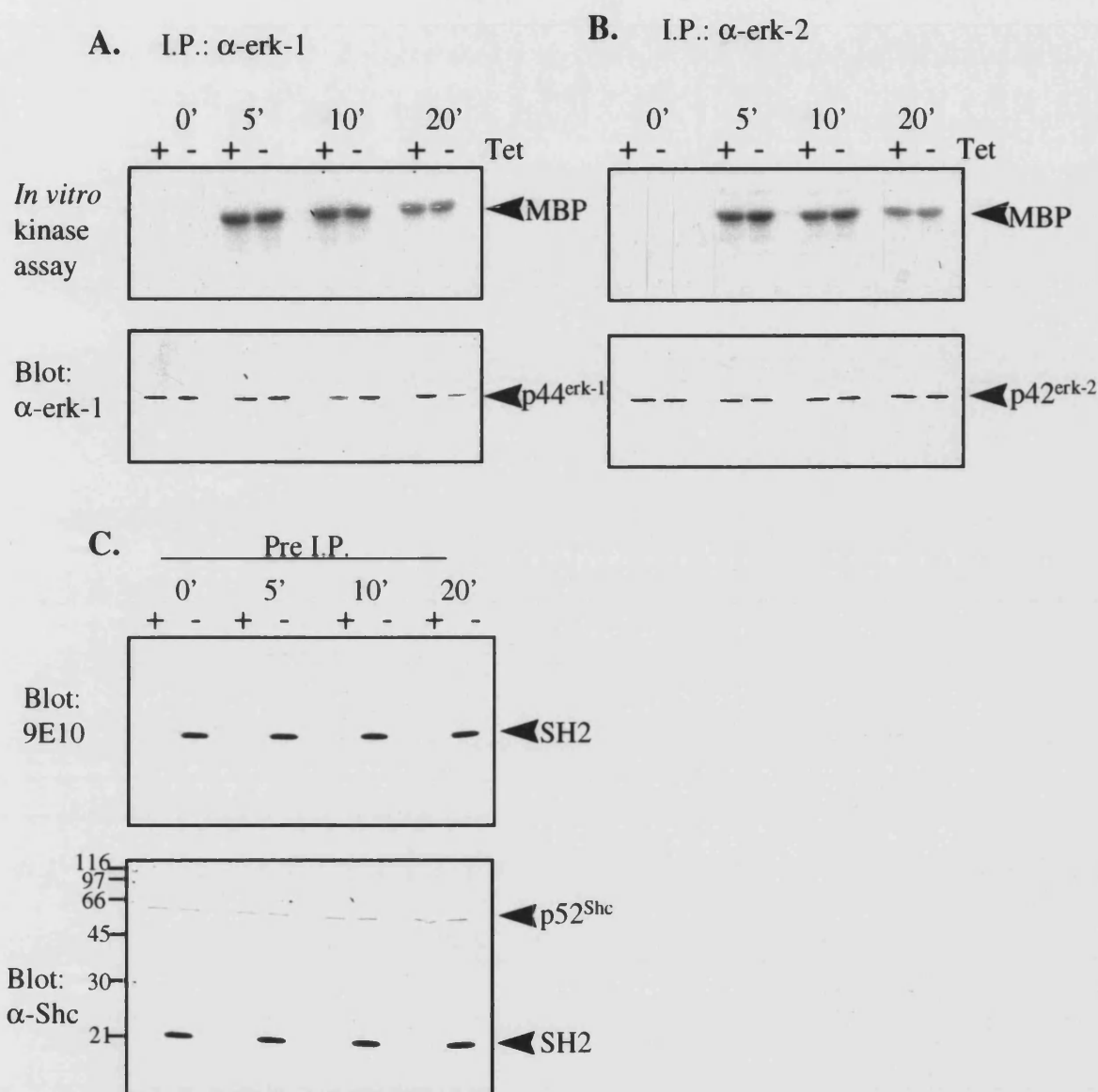
Clones Y317F 8D and FL 3E were plated at  $1 \times 10^5$  cell per ml in the presence (+) or absence (-) of 2  $\mu\text{g/ml}$  tetracycline for 16 hours. Factor deprived cells were then either left untreated as a control (C) or stimulated with IL-3 for 10 minutes (3). (A) Cell extracts from the equivalent of  $5 \times 10^6$  cells were precipitated with 10  $\mu\text{g}$  Grb2SH2-GST fusion protein. Samples were separated on a 7.5% gel and immunoblotted with 9E10 antibodies. (B) Samples of the total cell extracts were removed prior to precipitation ("Pre"), run on a separate 7.5% gel and immunoblotted with 9E10 antibodies. The positions of the molecular mass standards are shown and expressed in kDa and the positions of the expressed FL and Y317F Shc proteins are indicated. The results shown here are representative of three separate experiments.

In IL-3 stimulated cells expressing the Y317F mutant, 6-10 fold less expressed protein was precipitated by the SH2 domain of Grb2 than in cells expressing FL Shc (Fig. V.13 A), even though relatively equal amounts of Y317F Shc and FL Shc proteins were expressed (Fig. V.13 B). A small amount of Y317F Shc protein was precipitated by the Grb2SH2-GST fusion protein suggesting perhaps that Shc has other tyrosine(s) which can be inducibly tyrosine phosphorylated by IL-3 and that can interact with the SH2 domain of Grb2. However, even with this small amount of interaction, if the Ras/MAP kinase pathway was primarily and entirely activated by Shc binding to the receptor and subsequently localising Grb2 to the membrane by Y317 of Shc binding to the SH2 domain of Grb2, expression of the Y317F Shc mutant should substantially block this activation to a greater extent than was observed. This suggests that alternative pathways, not involving Shc, may function in activating the Ras/MAP kinase pathway in response to IL-3.

#### **V.G.4. Expression of the SH2 domain alone.**

Similar *in vitro* kinase assays as performed above (section V.G.1) were used to assess the effects of expression of the Shc SH2 domain on IL-3-induced activation of erk1 and erk2. Similar results were obtained for both SH2 expressing clones and the results from one clone are shown in Figure V.14. Expression of the SH2 domain alone had no apparent effect on IL-3-induced erk1 (Fig. V.14 A) or erk2 (Fig. V.14 B) activation, even though there was high expression of the SH2 protein (Fig. V.14 C) which was determined to be 50 fold that of endogenous p52<sup>Shc</sup>. More detailed dose-response (Fig. V.15 A) and time-course analyses (Fig. V.15 B) and immunoblotting with the anti-active MAP kinases antibody did not detect any effect on IL-3-induced erk1 or erk2 activation as a result of expression of the SH2 domain alone (as observed in two separate time-course and dose-response experiments), although good inducible expression was observed (lower panels).

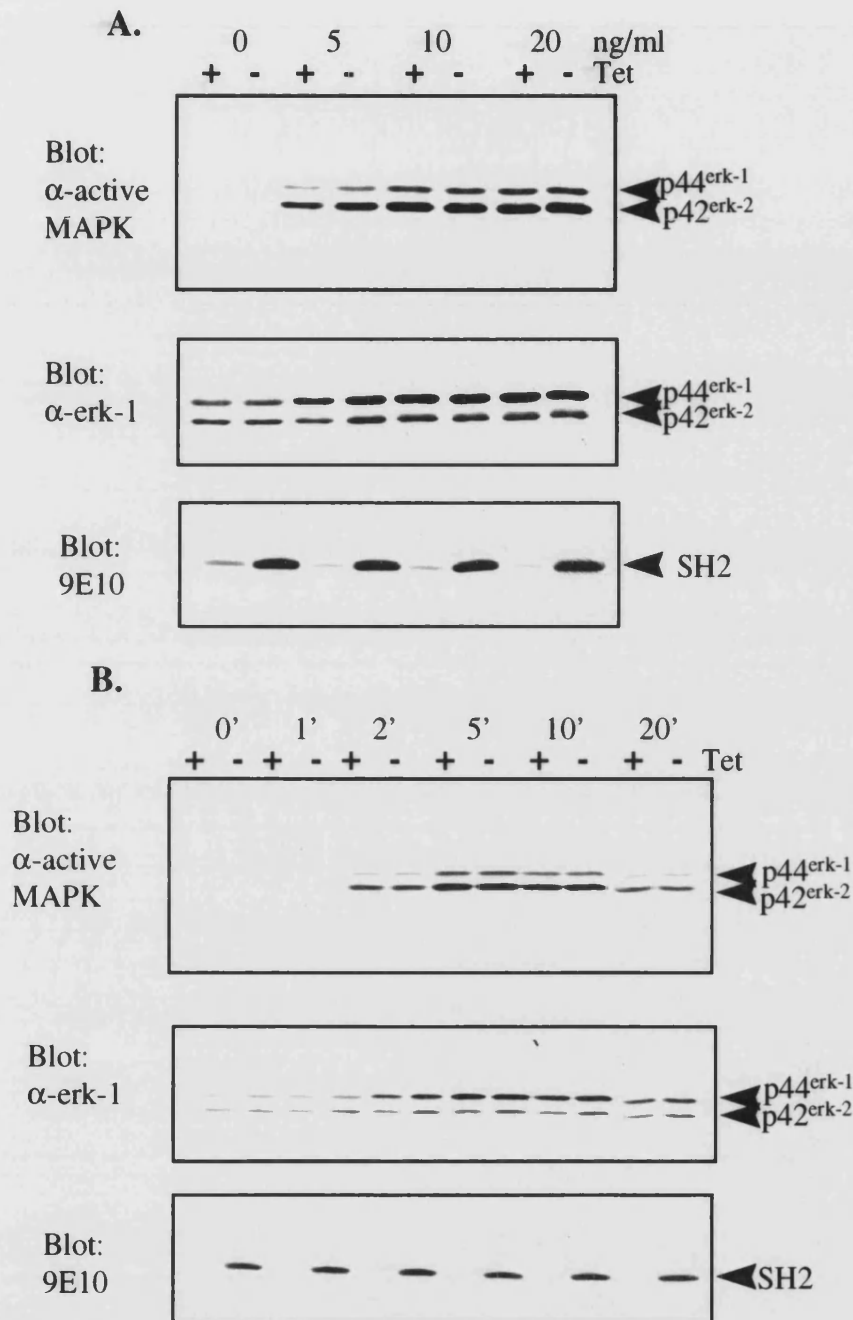
**FIGURE V.14**



**Expression of the SH2 domain alone does not have a significant effect on IL-3 induced MAP kinase activation in *in vitro* kinase assays.**

(A) Erk1 and erk2 (B) *in vitro* kinase assays were performed on clone SH2 3C11 as described in the legend to Figure V.9. Samples of the total cell extracts prior to precipitation were separated through a 12% acrylamide gel and immunoblotted with 9E10 antibodies for detection of the expressed protein (C upper panel), then stripped and reprobed with anti-Shc antibodies (C lower panel). The positions of erk1, erk2, p52<sup>Shc</sup> and the expressed SH2 Shc protein are indicated. Similar results were obtained with clone SH2 2A10.

**FIGURE V.15**



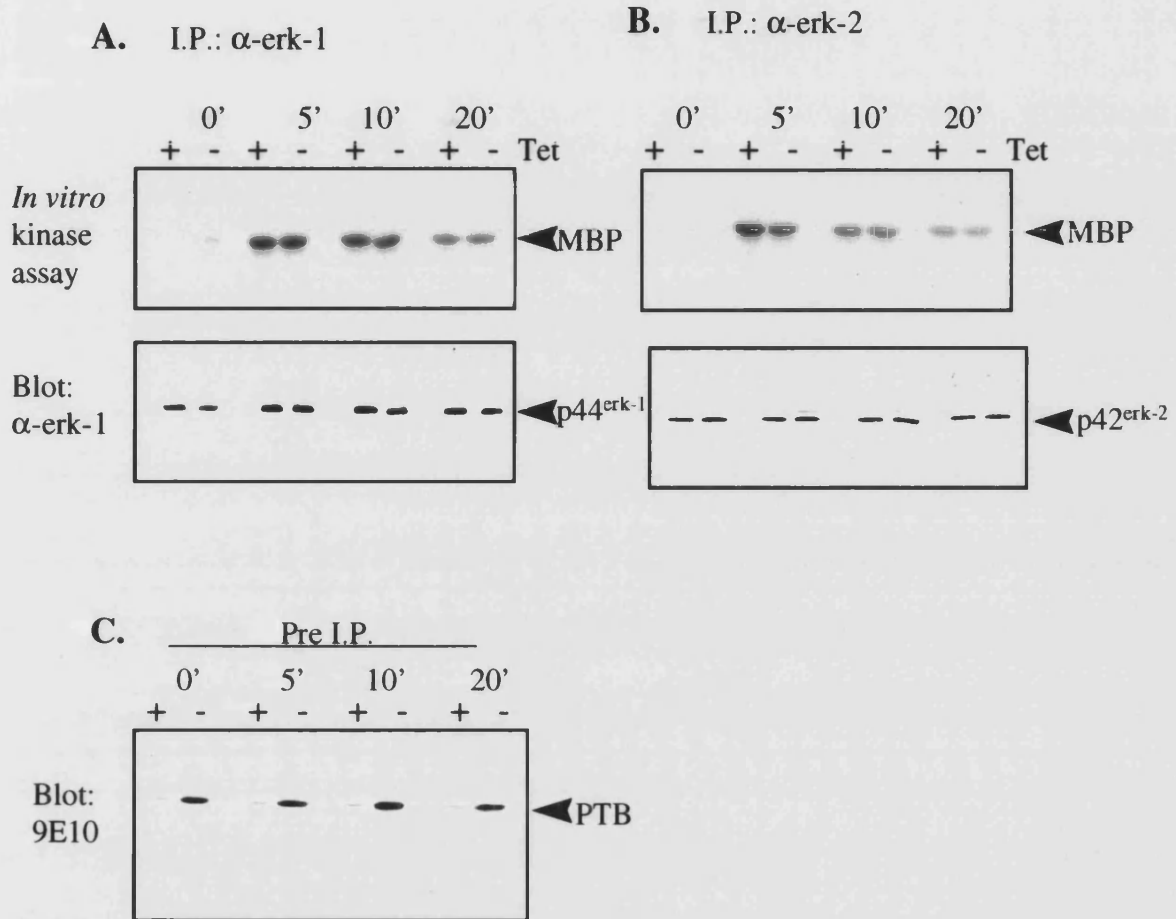
**Activation of erk1 and erk2 in response to IL-3 is not affected in cells expressing the SH2 domain alone.**

(A) IL-3 dose-response analyses were performed in clone SH2 3C11 and (B) time-course analyses were performed in clone SH2 2A10 as described in the legend to Figure V.10, except that samples were run on 12% acrylamide gels and immunoblotted with 9E10 antibodies to detect the expressed SH2 protein (A and B lower panels). The positions of the expressed SH2 protein, erk1, and erk2 are indicated. The data depicted here is representative of two separate time-course and dose-response experiments performed in both SH2 clones.

**V.G.5. Expression of the PTB domain alone.**

Finally, effects of expression of the Shc PTB domain alone on IL-3-induced erk1 and erk2 activation was investigated. *In vitro* kinase assays revealed no detectable effect on erk1 (Fig. V.16 A) or erk2 (Fig. V.16 B) activation upon expression of the Shc PTB domain. Good inducible expression of the PTB Shc protein was observed (Fig. V.16 C). No effect on IL-3-induced erk1 or erk2 activation were observed either after detailed dose-response (Fig. V.17 A) and time-course (Fig. V.17. B) analyses (as observed in four separate dose-response and two separate time-course experiments). Therefore, expression of the PTB domain alone also appears to have no detectable effect on IL-3-induced MAP kinase activation.

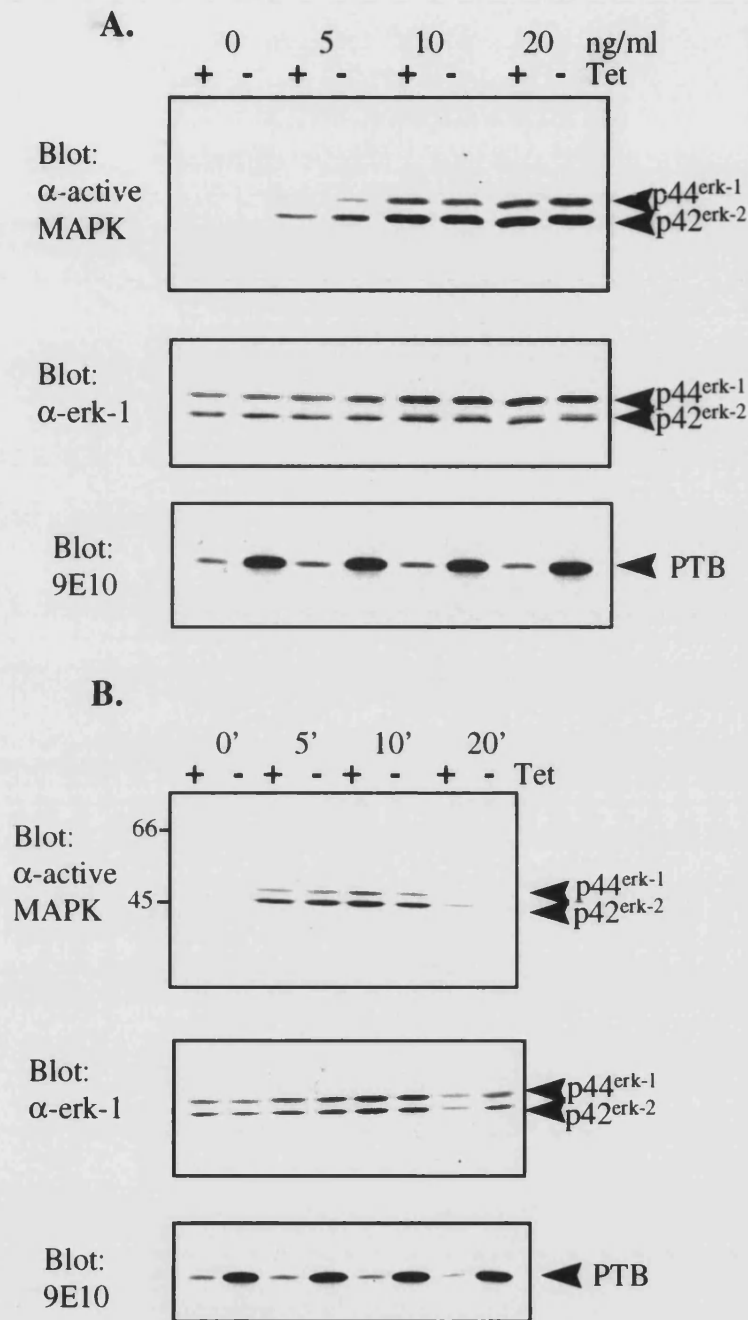
**FIGURE V.16**



**Expression of the PTB domain alone does not have a significant effect on IL-3 induced MAP kinase activation in *in vitro* kinase assays.**

(A) Erk1 and (B) erk2 *in vitro* kinase assays were performed on clone PTB 4F as described in the legend to Figure V.9. Samples of the total cell extracts prior to precipitation were separated through a 10% acrylamide gel and immunoblotted with 9E10 antibodies (C). The positions of erk1, erk2 and the expressed PTB protein are indicated. Similar results were obtained with clone PTB 4C.

**FIGURE V.17**



**Activation of erk1 and erk2 in response to IL-3 is not affected in cells expressing the PTB domain of Shc alone.**

(A) IL-3 dose-response and (B) time-course analyses were performed on clone PTB 4F as described in the legend to Figure V.10, except that samples were run on 12% acrylamide gels and immunoblotted with 9E10 antibodies (A and B lower panels). The positions of the expressed PTB protein, erk1 and erk2 are indicated. The data depicted here is representative of at least two separate experiments and similar results were observed for clone PTB 4C.

### **V.H. Effects of expression of Shc variants on proliferation in response to IL-3.**

Shc is thought to couple activated receptors to the Ras/MAP kinase pathway which regulates the proliferation of mammalian cells. Shc has been shown to have positive growth promoting activities, as constitutive overexpression of Shc in NIH3T3 mouse fibroblasts promotes a transformed phenotype in culture and the formation of tumours in nude mice (Pelicci *et al.*, 1992). Additionally, overexpression of Shc proteins has been shown to increase the response of TF-1 cells to GM-CSF (Lanfrancone *et al.*, 1995).

The biochemical changes observed in cells expressing the Shc variants in response to IL-3 were only moderate in the experiments performed. A decrease in endogenous Shc phosphorylation and minor effects on erk1 and erk2 activation were observed in cells expressing either FL or Y317F Shc. To assess what effects expressing the Shc variants have on the biological response to IL-3, the growth properties of the cells expressing the different Shc variants were investigated. The dose-response characteristics of the transfectants to IL-3 was measured using XTT assays. These assays are based on the reduction of XTT by NAD/NADPH oxidoreductases and are a measure of cellular metabolic activity and growth (Mosmann, 1983; Roehm *et al.*, 1991). The assays were performed in serum-free conditions, using AIM-V media, and used purified recombinant IL-3 so that only the effects specific to IL-3 were measured. The results of the XTT assays are depicted graphically in Figure V.18 along with the corresponding 9E10 immunoblots of whole cell lysates to show expression of the Shc variants over the 72 hour time period.

All proteins were inducibly and stably expressed at high levels through the duration (72 hr) of the XTT assays (see inserts to Fig. V.18 A-D). However, expression of FL Shc (Fig. V.18 A), the Y317F mutant (Fig. V.18 B), the SH2 domain alone (Fig. V.18 C), or the PTB domain alone (Fig. V.18 D) did not consistently have a significant effect on IL-3-induced proliferation as measured by these assays. Therefore, the biochemical effects observed with expressing the various Shc mutants (a decrease in endogenous Shc phosphorylation and changes in erk1 and erk2 activation) did not appear to detectably affect IL-3-dependent proliferation of the cells.

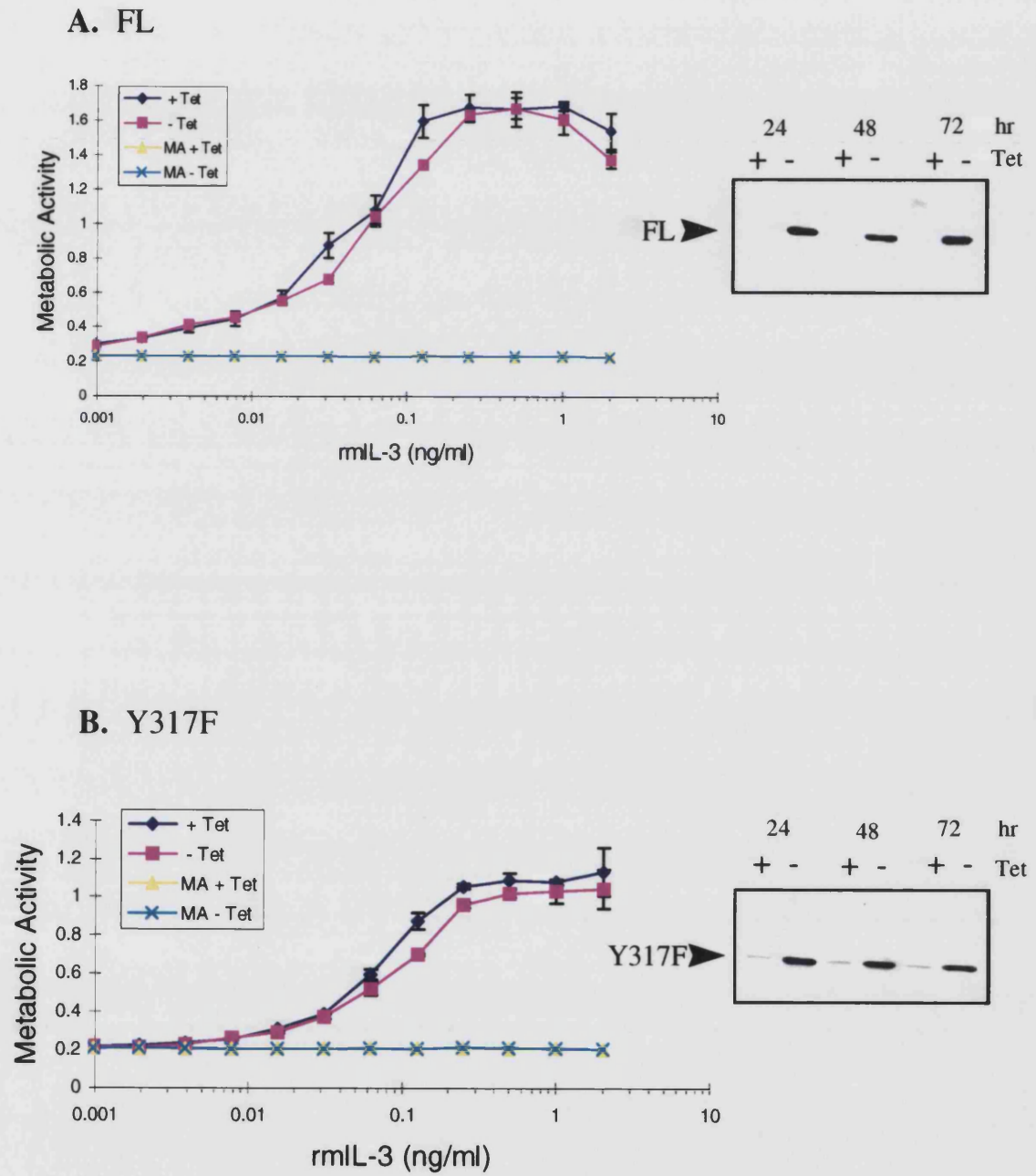


### **Figure V.18**

#### **Expression of the Shc variants has no affect on IL-3 responsiveness in XTT dye reduction assays.**

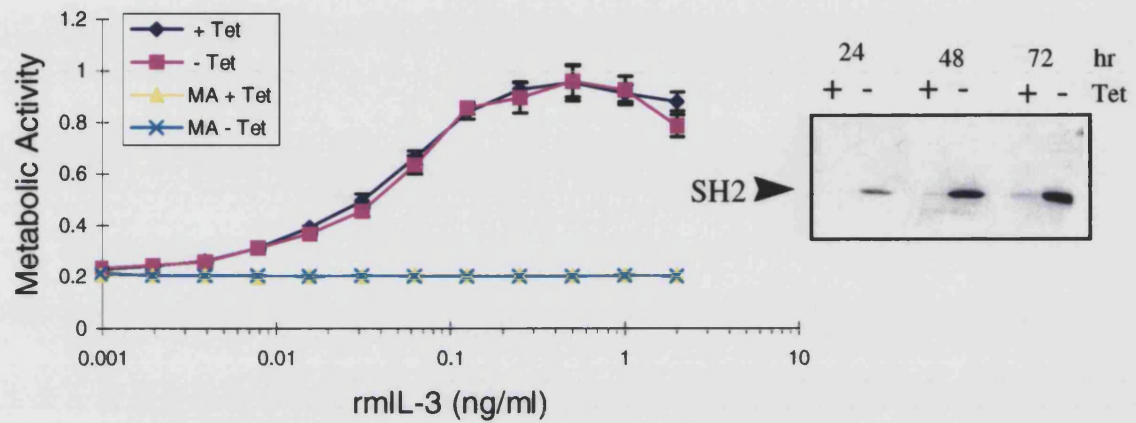
Recombinant IL-3 was set up at the doses shown in serum-free AIM-V media in triplicate. Clones FL 3E (A), Y317F 8D (B), SH2 3C11 (C) and PTB 4C (D) were plated at 1000 cells per well of a 96 well tray in the presence (diamonds) or absence (squares) of 2 µg/ml tetracycline. Cells were incubated for 72 hours with XTT being added to the cultures for the last 4 hours. Plates were read at 450 nm and the absorbance readings obtained equated to cellular metabolic activity. The mean values with standard deviations are plotted for each point. At the same time, clones were also set up at  $1 \times 10^4$  cells per ml (same concentration as the XTT assay) in the presence (+) or absence (-) of 2 µg/ml tetracycline and cell extracts prepared after 24, 48, and 72 hours. 15 µg of protein was loaded in each lane and separated through 7.5% (A and B), 12% (C) or 10% (D) acrylamide gels by SDS-PAGE and immunoblotted with 9E10 antibodies to detect the expressed protein (indicated). A representative experiment is shown in each case as the assays were repeated at least 3 times using both clones.

**FIGURE V.18**

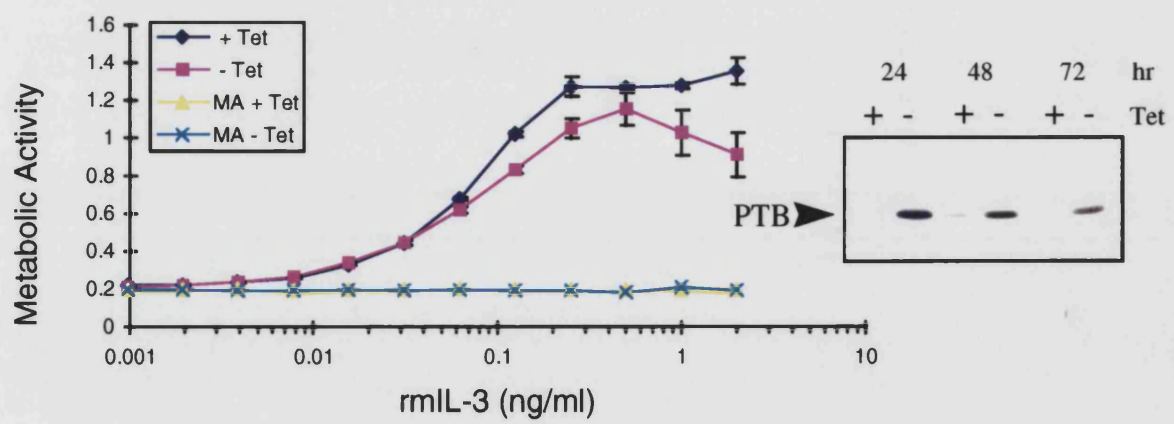


**FIGURE V.18**

**C. SH2**



**D. PTB**



## **V.I Discussion**

Following IL-3 treatment of factor-dependent myeloid cells, the p52 and p46 isoforms of Shc are two of the proteins which become most highly tyrosine phosphorylated (Cutler *et al.*, 1993; Welham *et al.*, 1994a). Shc proteins have primarily been implicated in controlling protein-protein interactions important for activation of the Ras/MAP kinase pathway (Rozakis-Addcock *et al.*, 1992; Salcini *et al.*, 1994). Evidence has been provided here that Shc can associate with the IL-3 receptor  $\beta$  subunit in human ( $\beta c$ ) and in murine (Aic2A) cells, via both its SH2 and PTB domains (section IV.E), providing two possible mechanisms for localising the Grb2-Sos complex to the plasma membrane, leading to activation of Ras. However, Shc can also bind via its PTB domain to SHIP (section IV.D) and via its SH2 domain to a tyrosine phosphorylated 100 kDa protein (section IV.F), thus potentially linking Shc to other signalling pathways involving PI3-K and lipid metabolism. Hence, to address the specific involvement of Shc in IL-3-dependent cell signalling, FL Shc, the Y317F mutant Shc, and the Shc SH2 and PTB domains individually were expressed in the IL-3-dependent Ba/F3 cell line.

The requirement of Shc in mediating IL-3-induced activation of the Ras/MAP kinase pathway could be investigated by comparing the effects of expressing full length Shc with a tyrosine to phenylalanine mutation at position 317 (the Grb2 SH2 binding site) with that of expressing FL Shc. Expression of the SH2 domain alone could block the interaction of endogenous Shc with other tyrosine phosphorylated signalling proteins, like p100, or block endogenous Shc from binding Aic2A which could have multiple consequences on IL-3 signalling events. Finally, expression of the Shc PTB domain alone could potentially block endogenous Shc from interacting with SHIP and/or the IL-3 receptor  $\beta$  subunit. The results presented here show that expression of both the FL Shc and the mutant Y317F Shc result in a consistent decrease in IL-3-induced endogenous Shc phosphorylation but, whereas expression of FL Shc appeared to slightly enhance erk1 and erk2 activation, expression of the Y317F mutant slightly decreased erk1 and erk2 activation. However, these biochemical changes did not appear to have in any detectable effects on IL-3-induced proliferation. Expression of the individual SH2 and PTB domains of Shc had no detectable effect on IL-3-induced tyrosine phosphorylation of endogenous

Shc or SHP-2, nor did their expression effect IL-3-induced erk1 or erk2 activation, or proliferation.

### **V.I.1 Endogenous Shc phosphorylation is reduced upon FL and Y317F Shc expression**

There is still some uncertainty as to whether Shc is required to bind to activated receptors in order to become tyrosine phosphorylated. Expression of the various Shc mutants had the potential to block endogenous Shc from binding to the IL-3 receptor and possibly result in a decrease in IL-3-induced endogenous Shc phosphorylation. Expression of the individual Shc SH2 or PTB domains had no detectable effect on IL-3-induced endogenous Shc phosphorylation but expression of FL Shc and the mutant Y317F Shc did result in a decrease in endogenous Shc tyrosine phosphorylation in response to IL-3. There is some evidence that tyrosine 577 of  $\beta c$  (the Shc PTB binding site) is required and sufficient for Shc phosphorylation (Durstin *et al.*, 1996; Itoh *et al.*, 1996; Okuda *et al.*, 1997) whereas phosphorylation of tyrosine 612 (the Shc SH2 binding site) is not required and not sufficient for Shc phosphorylation (Durstin *et al.*, 1996; Okuda *et al.*, 1997). These data are consistent with the view that the PTB domain of Shc is required to bind  $\beta c$  in order for Shc to become tyrosine phosphorylated. However, since the binding of Shc to  $\beta c$  was not examined in any of these studies, it still does not rule out the possibility that a kinase(s) needs to be recruited to tyrosine 577 of  $\beta c$  to phosphorylate Shc without requiring Shc itself to bind to the receptor. There is some evidence for this in EGF signalling as it appears that the binding of Shc to the EGFR is not required for phosphorylation of Shc as EGFR mutants lacking all autophosphorylation sites still induce tyrosine phosphorylation of Shc (Gotoh *et al.*, 1994; Soler *et al.*, 1994; Sasaoka *et al.*, 1996). Work by Gotoh *et al.* (1995) using Shc-GST fusion proteins in *in vitro* kinase assays with immunoprecipitated EGFR suggested that the Shc SH2 domain may regulate the phosphorylation of Shc by the EGFR tyrosine kinase. They suggest that the SH2 domain could inhibit phosphorylation of Shc through a mechanism of steric hindrance or local conformational change. Subsequent binding of the SH2 domain to a tyrosine phosphorylated protein could then release this hindrance, allowing for Shc to become tyrosine phosphorylated. Therefore, the reduction in IL-3-induced endogenous Shc phosphorylation observed when FL Shc and the Y317F mutant Shc protein were expressed may be due to the expressed proteins competing with

endogenous Shc for binding to Aic2A or alternatively competing for binding to other signalling molecules involved in pathways leading to Shc phosphorylation. The data presented here appears to support the notion that Shc does not need to bind to the receptor in order to become tyrosine phosphorylated as expression of the SH2 and PTB domains alone did not inhibit endogenous Shc phosphorylation. Work by Gotoh *et al.* (1996) had shown a reduction in IL-3-induced endogenous Shc phosphorylation when the SH2 domain of Shc was expressed, but this was most likely due to the expressed protein competing with endogenous Shc for a limited pool of anti-Shc antibodies, resulting in less endogenous Shc being precipitated from the cell, which they had not evaluated (see section V.F.2). However, there is also a possibility that the affinities of the SH2 and PTB domains individually are not high enough to displace or prevent endogenous Shc binding to the receptor and it has been previously shown that expression of the PTB and SH2 domains of Shc individually in NIH 3T3 cells overexpressing EGFR failed to inhibit endogenous Shc association with the activated EGFR (O'Bryan *et al.*, 1998).

SHP-2, like Shc, appears to interact via its SH2 domain with residues surrounding tyrosine 612 of  $\beta c$ . As with Shc, it has not been determined whether SHP-2 is required to bind to the receptor in order to become tyrosine phosphorylated. Expression of the Shc SH2 domain alone did not result in a decrease in IL-3-induced tyrosine phosphorylated SHP-2. If the Shc SH2 domain alone did not have high enough affinity to compete with SHP-2 for binding to Aic2A, one would perhaps expect that the FL Shc or Y317F mutant might have a high enough affinity. By stably binding to Aic2A via their PTB domains first and then binding, via their SH2 domains, to sequences surrounding tyrosine 610 of Aic2A, FL Shc and Y317F Shc could compete with SHP-2 for binding to Aic2A, therefore blocking SHP-2 phosphorylation. However, this was not observed, suggesting either that binding of SHP-2 to Aic2A is not a prerequisite for SHP-2 phosphorylation or that SHP-2 has a much higher binding affinity to Aic2A than Shc.

### **V.I.2 Involvement of Shc in IL-3-mediated activation of the Ras/MAP kinase pathway**

Shc has been implicated in regulating the activation of the Ras/MAP kinase pathway by mediating protein-protein interactions. Tyrosine phosphorylated Shc has been shown to form a complex with Grb2-Sos through the interaction of the SH2 domain of Grb2 with phosphorylated tyrosine 317 of Shc. This has been shown to be involved in

regulating activation of Ras, leading to erk1 and erk2 MAP kinase activity (Rozakis-Adcock *et al.*, 1992; Cutler *et al.*, 1993; Egan *et al.*, 1993; Skolnik *et al.*, 1993; Gotoh *et al.*, 1994; Salcini *et al.*, 1994). Therefore, it was somewhat surprising that a more pronounced effect on erk1 and erk2 activation, in response to IL-3, was not observed in cells expressing the Shc mutants. Expression of FL Shc slightly increased the rate of erk1 and erk2 activation by IL-3 at sub-maximal doses but did not affect the maximum levels (Fig. V.10). This is similar to what was seen in GM-CSF-stimulated TF-1 cells when p52 and p46 Shc were 5-fold overexpressed by retroviral-mediated gene transfer (Lanfrancone *et al.*, 1995). However, the effects observed in the TF-1 cells were more pronounced as overexpression of Shc increased the sensitivity to, enhanced the rate of, and prolonged GM-CSF induced MAP kinase activation (Lanfrancone *et al.*, 1995). However, constitutive overexpression of Shc could result in selecting out a population of cells, whereas with the tetracycline-regulated system, one would hope to avoid this since cells are normally grown in tetracycline to repress expression.

Tyrosine 317 of Shc is the primary site of phosphorylation and the binding site for the Grb2 SH2 domain. Thus, expression of the Y317F mutant Shc protein would potentially interfere with activation of the Ras/MAP kinase pathway. However, expression of the Y317F Shc mutant resulted only in a slight decrease in IL-3-stimulated erk1 and erk2 activation (Fig. V.12). However, in agreement with the results presented here, activation of MAP kinase was not observed to be affected in GM-CSF-stimulated TF-1 cells constitutively expressing a Y317F Shc mutant (Lanfrancone *et al.*, 1995). In addition, only a slight down-regulation in EGF-induced MAP kinase activation was also reported in 293 T cells transiently expressing Y317F Shc (Thomas *et al.*, 1997). This is contradictory to what was observed by Gotoh *et al.* (1997) where expression of Y317F Shc caused a dramatic decrease in EGF-stimulated MAP kinase activation in NIH 3T3 cells expressing autophosphorylation site-defective mutant EGFR. This mutant EGFR was truncated after residue 1011, thus removing the major autophosphorylation sites, and a minor phosphorylation site, Y992, was mutated to phenylalanine (Gotoh *et al.*, 1994). This receptor mutant was previously shown to induce EGF-stimulated tyrosine phosphorylation of Shc, resulting in complex formation with Grb2 and activation of MAP kinase (Gotoh *et al.*, 1994). Thus, in this system, it appears that phosphorylation of Shc on tyrosine 317 is

required for activation of the Ras/MAP kinase pathway, but direct binding to the EGFR is not necessary.

Recently, two novel tyrosine phosphorylation sites in Shc, Y239/240, have also been shown to become phosphorylated in response to IL-3 (Gotoh *et al.*, 1996), EGF (Gotoh *et al.*, 1997) and mT-transformation (Blaikie *et al.*, 1997) and may be involved in coupling Shc to Grb2. Sequence alignment of Shc proteins with other adaptor proteins displaying a high level of identity in their PTB and SH2 domains have revealed that tyrosines 239 and 240 are well conserved (Nakamura *et al.*, 1996; O'Bryan *et al.*, 1996; van der Geer *et al.*, 1995). In *Drosophila*, Shc lacks a tyrosine at the position comparable to tyrosine 317 in mammalian Shc (Lai *et al.*, 1995) and phosphorylation of tyrosine 239 of Shc does create a potential consensus sequence (pYYND) for the binding of the SH2 domain of Grb2. There have been reports that Grb2 can bind to the sites surrounding tyrosine 239 of Shc. In EGF-stimulated 293 T cells it has been shown that a GST-ShcY317F fusion protein was strongly tyrosine phosphorylated and associated with Grb2 following EGF stimulation whereas a GST-Y239/240F Shc fusion protein (in which tyrosines 239 and 240 had been changed to phenylalanine residues) was only weakly tyrosine phosphorylated and only weakly bound Grb2, suggesting that tyrosines 239/240 are the major site of tyrosine phosphorylation and Grb2 binding (Thomas *et al.*, 1997). Furthermore, in mT-transformed fibroblasts, Y239/240 were also found to be the major tyrosines phosphorylated and created a Grb2 binding site whereas tyrosine 317 of Shc was not detectably tyrosine phosphorylated (Blaikie *et al.*, 1997). However, IL-3-stimulated MAP kinase activity has been demonstrated to be enhanced in cells expressing Y239/240F mutant but was slightly decreased in Y317F expressing cells, suggesting that Y239/240 are not involved in activation of the Grb2/mSos/Ras pathway by IL-3 (Gotoh *et al.*, 1996). Additionally, EGF-induced erk1 and erk2 activation was efficiently activated to similar levels in cells expressing a Y239/240F mutant compared with cells expressing FL Shc (Gotoh *et al.*, 1997). In cells expressing the Y317F Shc mutant, a weak binding of Grb2 to the Y317F Shc protein was observed by Gotoh *et al.* (1997), similar to what was observed in this study (Fig. V.13). However, this low level of Grb2 binding did not contribute to EGF-induced Ras/MAP kinase activation as erk1 and erk2 activation were dramatically decreased to undetectable levels in cell expressing the Y317F Shc mutant (Gotoh *et al.* 1997), contrary to the only slight decrease in IL-3 stimulated erk1 and erk2 activation



observed in this study. Thus, the low level of Grb2 binding to the Y317F Shc protein (see Fig. V.13) was unlikely to contribute significantly to the activation of erk1 and erk2. It is possible that in some systems tyrosines 239/240 may be the major tyrosine phosphorylation and Grb2 binding site, whereas in other systems, tyrosine 317 is the major player. However, since little tyrosine phosphorylation or binding to the Grb2 SH2 domain of the expressed Y317F Shc mutant was observed here and Y239/240 have been shown not to be involved in activation of the Ras/MAP kinase pathway in response to IL-3 (Gotoh *et al.*, 1997), this seems to imply that phosphorylation of Shc in IL-3 stimulated Ba/F3 cells occurs primarily at tyrosine 317, providing the major Grb2 binding site.

Since the PTB domain of Shc appears to have a higher affinity for Aic2A than the SH2 domain (section IV.E), binding of Shc via its PTB domain to the receptor may play a more significant role in localising Grb2-Sos complex to the membrane than the Shc SH2 domain. However, expression of the PTB domain alone also did not lead to a reduction in IL-3-stimulated erk1 or erk2 activation (Fig. V.16 and V.17). The PTB domain of Shc has been shown to bind with higher affinity to the activated EGFR compared with the SH2 domain (Sakaguchi *et al.*, 1998) and expression of the PTB domain alone also did not inhibit EGF-activated erk2 phosphorylation (O'Bryan *et al.*, 1998). This again raises the possibility that perhaps the affinities of the expressed SH2 and PTB domains alone were not high enough to displace or compete with endogenous Shc or that the cells can utilise alternative signalling pathways towards IL-3 activation of the MAP kinases erk1 and erk2. Alternatively, the expressed Shc PTB domain could bind to SHIP and therefore prevent endogenous Shc from binding SHIP which may result in a more pronounced effect on SHIP phosphatase activity and lipid metabolism in response to IL-3. However, this was beyond the scope of the work presented here.

Shc may not be the primary pathway towards activation of Ras in haemopoietic cells in response to IL-3. Indeed, recently Shc has been shown to be dispensable for B cell antigen receptor (BCR)-induced erk activation in DT40 B cells (Hashimoto *et al.*, 1998). BCR stimulation stimulation has also been shown to lead to tyrosine phosphorylation of Shc and to the assembly of Shc-Grb2-Sos complexes. However, Shc-deficient DT40 B cells exhibit normal BCR-induced erk activation, whereas this erk activation was inhibited by loss of Grb2 or expression of dominant negative Ras (Ras N17) (Hashimoto *et al.*, 1998). Thus, alternative, Shc-independent pathways may be responsible for activation of

the Ras/MAP kinase pathway in response to IL-3. SHP-2 is tyrosine phosphorylated in response to IL-3 and can associate with Grb2 (Welham *et al.*, 1994b). SHP-2 has been shown to bind  $\beta c$  (Chapter III and Bone *et al.*, 1997) suggesting that SHP-2 could act as an adaptor between activated  $\beta c$  and Grb2, thus leading to activation of the Ras/MAP kinase pathway. Since both SHP-2 and Shc have been shown in these studies to interact via their SH2 domains with residues surrounding tyrosine 612 of  $\beta c$ , expression of the Shc SH2 domain alone could potentially compete with both endogenous Shc and SHP-2 for binding to Aic2A and therefore potentially block two pathways leading to the activation of Ras and subsequently erk1 and erk2. However, a reduction in IL-3-induced erk1 and erk2 activation was not observed in cells expressing the Shc SH2 domain (Fig. V.14 and V.15). Gotoh *et al.* (1996) also reported only a moderate inhibition of IL-3-stimulated MAP kinase activity upon expression of the Shc SH2 domain alone in Ba/F3 cells. However, overexpression of the Shc SH2 domain has been shown to block 50-70% of EGF-induced MAP kinase activation (Thomas *et al.*, 1997; O'Bryan *et al.* 1998). The regulation of the Ras/MAP kinase pathway by SHP-2 has been suggested in other systems where SHP-2 acts as an adaptor between Grb2 and c-kit (Tauchi *et al.*, 1994), the EpoR (Tauchi *et al.*, 1995) and the PDGFR (Li *et al.*, 1994; Bennett *et al.* 1994). In response to insulin, expression of a catalytically inactive mutant SHP-2 (Noguchi *et al.*, 1994) or dominant interfering mutants of SHP-2 (Yamauchi *et al.*, 1995) were also found to inhibit MAP kinase activation. Recently, SHP-2 was demonstrated to perform an essential role in EGF-stimulated MAP kinase activation (Deb *et al.*, 1998). The enzymatic activity and both the nSH2 and cSH2 domains of SHP-2 were found to be required for MAP kinase activation as transfection of a truncated form of SHP-2 containing only the two SH2 domains or transfection of a catalytically inactive SHP-2 blocked EGF-stimulated activation of transfected MAP kinase in COS7 cells (Deb *et al.*, 1998). However, these dominant negative forms of SHP-2 had no effect on EGF-stimulated interaction of Grb2 with the EGFR or Shc, nor did they influence phosphorylation of Shc or Shc-EGFR association (Deb *et al.*, 1998). Therefore, SHP-2 function, but not Shc, appears to be essential for EGF-induced MAP kinase activation, which may also be the case in response to IL-3. Indeed, recently it has been shown that expression of a dominant negative SHP-2 mutant, with a deletion in the catalytic domain, in Ba/F3 cells inhibits IL-3-induced MAP kinase activation (Gu *et al.*, 1998).

In addition to SHP-2, PI3-K has also been shown to be involved in activation of the Ras/MAP kinase pathway. The p110 catalytic subunit of PI3-K has been shown to directly interact with Ras (Rodriguez-Viciana *et al.*, 1994). Initially, the PI3-K inhibitor, wortmannin, was shown to inhibit activation of the MAP kinases erk1 and erk2 in response to T cell receptor stimulation (Von Willebrand *et al.*, 1996) and insulin (Welsh *et al.*, 1994; Cross *et al.*, 1994). More recently, expression of dominant negative PI3-K mutants which lack the p110 catalytic subunit binding site ( $\Delta p85$ ), have been shown to inhibit erk1 and erk2 activation after T cell receptor stimulation (Jascur *et al.*, 1997) and in response to IL-3 (Craddock, personal communication). Therefore, there appears to be multiple pathways leading to the activation of erk1 and erk2 and disruption of one of these pathways may not significantly affect activation of erk1 and erk2 as another pathway could take over. Thus the importance of the Ras/MAP kinase pathway in cellular proliferation and survival could be reflected in the adaptation of redundant pathways.

### **V.I.3 Requirement of Shc for IL-3-dependent proliferation**

Expression of the Shc mutants only had small effects on the biochemical events studied. However, Shc has been shown to be important for stimulating the intracellular transmission of growth and differentiation signals. Overexpression of Shc proteins in cultured fibroblasts has been shown to induce a transformed phenotype in culture and form tumours in nude mice (Pelicci *et al.*, 1992) and when overexpressed in PC12 cells, Shc has been shown to induce neurite outgrowth which is dependent on Ras activation (Rozakis-Adcock *et al.*, 1992). However, in this study, none of the Shc mutants expressed had a significant effect on IL-3-induced proliferation, again suggesting the possibility of redundant pathways. EGF-stimulated growth has been shown to be inhibited by expression of the Y317F Shc mutant (Gotoh *et al.*, 1997) as well as by expression of the Shc SH2 and PTB domains alone (O'Bryan *et al.*, 1998). Other investigators have microinjected various Shc-GST constructs to probe Shc function in fibroblasts. Microinjection of the isolated Shc SH2 domain inhibited DNA synthesis induced by both EGF (Gotoh *et al.*, 1995; Ricketts *et al.*, 1996; Sasaoka *et al.*, 1996) and PDGF (Roche *et al.*, 1996) but not insulin (Ricketts *et al.*, 1996; Sasaoka *et al.*, 1996). Insulin-induced DNA synthesis was inhibited by microinjection of the Shc PTB alone (Ricketts *et al.*, 1996; Sasaoka *et al.*, 1996). The PTB domain of Shc also inhibited EGF-induced mitogenic signals but not as efficiently as

the SH2 domain (Ricketts *et al.*, 1996; Sasaoka *et al.*, 1996). However, the levels of protein microinjected into the cells is likely to be very high and many of these experiments were performed in fibroblasts overexpressing the appropriate receptors, questioning the physiological relevance of these experiments. The experiments presented in this thesis utilise an inducible expression system, which results in 10-50 fold expression of the Shc mutants compared with endogenous p52<sup>Shc</sup>. IL-3-dependent haemopoietic cells were also used which do not overexpress IL-3 receptors, thus making this system more physiologically relevant.

#### V.I.4 General Observations

Several possibilities exist for the limited effects observed on IL-3-induced signalling upon expression of the Shc mutants, particularly the individual PTB and SH2 domains. Firstly, the SH2 and PTB domains individually may not be able to bind with high enough affinity individually to compete with endogenous Shc for binding to the receptor or other downstream signalling molecules. However, expression of the individual Shc SH2 domain has been shown to be able to block EGF-induced MAP kinase activation and growth and expression of the Shc PTB domain alone was also able to inhibit EGF-stimulated growth (Thomas *et al.*, 1997; O'Bryan *et al.*, 1998). Alternatively, the expression level of the Shc mutants may be an important factor in inhibiting signalling pathways. In microinjection experiments, Gotoh *et al.*, (1995) observed a decrease in EGF-stimulated DNA synthesis when cells were injected with 4-8 mg/ml Shc SH2 but noted at 0.5 mg/ml there was only a marginal effect on DNA synthesis. However, the expression level of the Shc mutants here were almost identical to  $\Delta p85$  mutants expressed in Ba/F3 cells where clear effects on IL-3-induced signalling pathways were observed (Craddock *et al.*, 1999). Thus, the 10-50 fold overexpression of the Shc variants typically seen in the experiments presented here should be substantial enough to block Shc-dependent IL-3 signalling pathways. In addition, the expressed Shc proteins could be detected interacting with tyrosine phosphorylated proteins (see Fig. V.4) and the expressed Shc proteins were stably expressed (see section V.D). Therefore, the most likely reason for the moderate effects observed on IL-3-induced signalling pathways upon overexpression of the Shc mutants is that Shc-mediated pathways are not essential for IL-3-induced activation of the Ras/MAP kinase pathway or for IL-3-induced signals leading to

proliferation in haemopoietic cells, emphasising redundancy and cross-talk within the system.

The data presented here do not support the premise that Shc is an essential and indispensable protein important in the generation of signalling events downstream of the IL-3 receptor, despite Shc proteins being one of the major tyrosine phosphorylated substrates in response to IL-3. Shc may play a role in a signalling pathway leading to erk1 and erk2 activation. However, there appears to be redundancy in the signal transducing pathways leading to activation of the Ras/MAP kinase pathway and ultimately to growth and differentiation in IL-3-dependent signalling in haemopoietic cells, highlighting the importance of this pathway in proliferation due to the existence of multiple pathways leading to its activation.

**Chapter VI**  
**General Discussion**

The cytokine IL-3 is an important regulator of haemopoiesis by acting as a growth, survival, and differentiation factor for a broad range of haemopoietic cells including pluripotent stem cells and progenitors, mast cells, megakaryocytes, macrophages, neutrophils, and basophils (Arai *et al.*, 1990; Ihle *et al.*, 1992). Although both  $\alpha$  and  $\beta$  subunits of the IL-3 receptor lack intrinsic tyrosine kinase activity, IL-3 treatment induces tyrosine phosphorylation of the  $\beta$  subunit of its receptor on multiple tyrosine residues which are potential sites of interaction for the SH2- and PTB- domain containing proteins. A number of signalling proteins including SHIP (Damen *et al.*, 1996), Jak-2 (Silvennoinen *et al.*, 1993), STAT5 (Mui *et al.*, 1995), SHP-2 (Welham *et al.*, 1994b), the MAP kinases erk1 and erk2 (Welham *et al.*, 1992), and the two Shc isoforms p52<sup>Shc</sup> and p46<sup>Shc</sup> (Cutler *et al.*, 1994; Welham *et al.*, 1994a) are also inducibly tyrosine phosphorylated in response to IL-3 stimulation. However, the functional significance of many of these events in IL-3-induced proliferation, survival, and differentiation of haemopoietic cells has not been determined.

In this study, the functional role of the PTPases, SHP-1 and SHP-2, and the adaptor protein, Shc, in IL-3 signalling was examined. This was accomplished using a combination of biochemical, genetic, and cellular techniques. The protein-protein interactions mediated by SHP-1, SHP-2, and Shc and their various domains individually, identified potential roles for these signalling molecules in integrating IL-3 signals. *In vitro* binding studies demonstrated that the SH2 domains of SHP-1, SHP-2 and Shc all bound directly to the phosphorylated IL-3 receptor  $\beta$  subunit via residues surrounding tyrosine 612/610. Shc was also able to directly interact with residues surrounding a different tyrosine within the  $\beta$  subunit of the IL-3 receptor (Y577 in  $\beta_c$  and Y575 in Aic2A) via its PTB domain. Thus, these results suggested that these molecules may have regulatory roles at the receptor itself or may function as adaptor molecules, localising other signalling molecules to the receptor and so to the plasma membrane and the vicinity of their substrates. Indeed, both SHP-2 and Shc have been implicated in regulation of the Ras/MAP kinase pathway. The binding of Grb2 to SHP-2 and/or Shc and the association of SHP-2 and Shc with the IL-3 receptor  $\beta$  subunit could provide a means of localising Grb2-associated Sos to the plasma membrane where it could activate Ras. Alternatively, both SHP-2 and Shc appear to also associate with a 100 kDa protein which binds the p85 subunit of PI3-K, so localisation of SHP-2 and/or Shc may function in activating pathways

mediated by PI3-K. However, SHP-2 and Shc may also function in localising SHIP to the plasma membrane, possibly leading to the down-regulation of PI3-K activated pathways, due to the ability of SHIP to dephosphorylate the primary product of PI3-K, PI(3,4,5)P<sub>3</sub>, or down-regulate the Ras/MAP kinase pathway by competing with Grb2 for binding to Shc and/or SHP-2. Complicating the matter still, SHP-1 and SHP-2 may be able to control these interactions themselves as they both appear to be able to dephosphorylate the receptor at tyrosine 612/610, the binding site for the SH2 domains of SHP-1, SHP-2 and Shc.

Recently, the physiological functions of SHP-2 have been investigated in mammals by introducing a targeted mutation into the murine *SHP-2* locus resulting in an internal deletion from amino acid 46 to 100 in the amino-terminal SH2 domain (Saxton *et al.*, 1997). Homozygous mutant (*SHP-2*<sup>-/-</sup>) mice died mid-gestation with severe defects in mesodermal patterning (Saxton *et al.*, 1997). A similar phenotype was also observed in *Xenopus* embryos through microinjection of catalytically inactive mutant mRNA of SHP-2 (Tang *et al.*, 1995). By isolating mutant *SHP-2*<sup>-/-</sup> ES cell lines, the effect of the SHP-2 mutation on haemopoietic cell differentiation of ES cells was assessed (Qu *et al.*, 1997). The mutation introduced into the *SHP-2* locus resulted in severe suppression of development of erythroid progenitors and completely blocked production of progenitor cells for granulocytes-macrophages and mast cells (Qu *et al.*, 1997). Interestingly, MAP kinase activity induced by SLF, which serves as a growth factor for erythroid and myeloid cells, was blocked in homozygous (*SHP-2*<sup>-/-</sup>) mutant ES cells (Qu *et al.*, 1997). Additionally, in *SHP-2*<sup>-/-</sup> mutant embryonic fibroblast cell lines, erk activation by EGF and PDGF was attenuated and IGF-1-induced erk activation was also completely blocked (Shi *et al.*, 1998). Therefore, these results suggest that physiologically, SHP-2 plays an important role in the development of haemopoietic cells and acts as a positive regulator in mitogenic signalling pathways leading to erk activation.

Given that Shc is thought to play an important role in controlling activation of the Ras/MAP kinase pathway, the physiological significance of the protein-protein interactions mediated by Shc were determined in this study by expressing Shc mutants in an IL-3-dependent cell line and examining their effects on IL-3-induced MAP kinase activation and proliferation. Expression of FL Shc only slightly enhanced the rate of erk1 and erk2 activation and expression of a Shc mutant with tyrosine 317, the Grb2 binding site, mutated to phenylalanine only slightly reduced IL-3-induced MAP kinase activation. Additionally,



expression of none of the Shc mutants had detectable effects on IL-3-induced proliferation. Thus, even though Shc becomes highly tyrosine phosphorylated in response to IL-3, it does not appear to be an essential and indispensable protein important for generating signalling events downstream of the IL-3 receptor. Perhaps this is not surprising considering the pleiotropic activity of IL-3 and the importance of the Ras/MAP kinase pathway in controlling proliferation. Therefore, it would make sense for the cell to have more than one mechanism for controlling activation of the Ras/MAP kinase pathway. Indeed, in this system, SHP-2 also appears to have similar adaptor-like functions as Shc and may also be involved in regulation of this pathway.

Shc may have an alternative role in maintaining cell viability. It has been suggested that Shc may be involved in the anti-apoptotic activity of IL-3 (Kinoshita *et al.*, 1995). Phosphorylation of Shc at tyrosines 239/240 appears to have a role in induction of *c-myc*, in a Ras/MAP kinase-independent manner, leading to suppression of apoptosis (Gotoh *et al.*, 1996). Under two conditions in which cells are prone to be apoptotic, in the absence of IL-3 but with sufficient serum and in the presence of IL-3 but with low serum, Ba/F3 cells expressing FL Shc and the Y317F mutant Shc survived longer than Y239/240F Shc-expressing cells (Gotoh *et al.*, 1996). Therefore, Shc may have a role in anti-apoptotic pathways and in combination with activation of the Ras/MAP kinase pathway may lead to optimum IL-3-induced mitogenic signalling. Mutation of tyrosines 239/240 to phenylalanine alone, or in combination with mutation of tyrosine 317, would be interesting to explore in the system. Perhaps Shc plays a more important role in survival in Ba/F3 cells.

Since both SHP-2 and Shc may have similar roles as adaptor proteins in integrating IL-3 signalling events leading to activation of the Ras/MAP kinase pathway, the contribution each of these pathways makes towards activation of the Ras/MAP kinase pathway should be investigated. Initially, the effects of expressing various SHP-2 mutants, including a catalytically inactive mutant and mutants expressing the two SH2 domains or the individual SH2 domains alone, on IL-3-induced MAP kinase activation would be interesting to explore. If a dramatic effect on erk activation was observed with these mutants, it could suggest that the SHP-2 plays a dominant role in regulating IL-3 signalling pathways leading to activation of MAP kinase. Alternatively, if only moderate effects were observed on erk activation in cells expressing SHP-2 mutants, it would be interesting to

also express the Shc mutants in the SHP-2 mutant expressing cells and examine if this has a more dramatic effect on IL-3-induced erk activation and proliferation.

Since both SHP-2 and Shc appear to bind to residues surrounding the same tyrosine within  $\beta c/Aic2A$ , the specific roles of tyrosine 612/610 as well as tyrosines 577/575 and 750/745 in mediating IL-3 signalling events could also be investigated. By mutation of these tyrosine residues, not only could the association of SHP-2 and Shc with the mutant IL-3 receptors be investigated but also the consequences on downstream signalling events such as tyrosine phosphorylation of SHP-2 and Shc themselves, activation of the Ras/MAP kinase pathway and SHIP activity. I have generated these mutants and they are ready to be transfected into cells already expressing the mouse IL-3 receptor  $\alpha$  chain.

Clearly, the signalling events following IL-3 stimulation, leading to activation of the Ras/MAP kinase pathway and ultimately cell proliferation, and survival are becoming increasingly complex. The observations presented in this study that expression the Shc mutants showed little IL-3-induced biochemical or biological effects strongly emphasises the cell's adaptation of redundancy and cross-talk between signalling pathways to ensure propagation of signals important for cell survival and proliferation. Indeed, it is suggested in this study that SHP-2 may also function in a similar pathway, leading to erk1 and erk2 activation, since it associates with the same phosphotyrosine residue, tyrosine 612/610 of  $\beta c/Aic2A$ , as does Shc, and can also act as an adaptor protein, linking Grb2-Sos complexes to the receptor. Thus, expression of receptor mutants, lacking critical tyrosine residues together with studies examining the effects of expressing SHP-2 mutants will help to determine the requirement or redundancy of the various pathways leading to erk activation and ultimately cell proliferation and survival in response to IL-3.

**APPENDIX I**  
**Statistical Analysis**

Analysis can be performed on biochemical results to determine the statistical relevance of the particular observations. This usually involves scanning and densitometric analysis of immunoblots from at least three different experiments. Here, the fold decrease in endogenous p52<sup>Shc</sup> tyrosine phosphorylation upon expression of FL Shc and Y317F Shc in whole cell extracts after 10 minutes of IL-3 stimulation (refer to Fig. V.5 A and B) and precipitated by Grb2SH2-GST (refer to Fig. V.7 A and B) were calculated and the statistical significance determined.

Immunoblots were developed by the ECL chemiluminescent detection system and Kodak XAR-5 film was used for the detection of ECL signals. The film from three individual experiments were then scanned on a BioRad GS-670 imaging densitometer and volumes of the tyrosine phosphorylated p52<sup>Shc</sup> bands determined. The ratio of p52<sup>Shc</sup> band intensity from cells not expressing FL or Y317F Shc (+ tetracycline sample) compared to those expressing the Shc constructs (- tetracycline samples) were calculated. The volumes and ratios determined from the various experiments are outlined in Tables VII.1 and VII.2

**Table VII.1**

**Volumes and calculated ratios of endogenous p52<sup>Shc</sup> tyrosine phosphorylated bands in whole cell extracts**

	FL Shc			Y317F Shc		
	Exp.#1	Exp.#2	Exp.#3	Exp.#1	Exp.#2	Exp.#3
<b>Vol.</b> +Tet	41.35	78.09	59.71	33.10	58.70	60.35
-Tet	11.46	21.91	40.29	14.09	41.30	39.65
<b>Ratio</b>	<b>3.61</b>	<b>3.56</b>	<b>1.48</b>	<b>2.35</b>	<b>1.42</b>	<b>1.52</b>

**Table VII.2**

**Volumes and calculated ratios of endogenous p52Shc tyrosine phosphorylated bands precipitated by Grb2SH2-GST**

	FL Shc			Y317F Shc		
	Exp.#1	Exp.#2	Exp.#3	Exp.#1	Exp.#2	Exp.#3
<b>Vol. +Tet</b>	54.39	40.50	23.17	28.63	21.24	55.96
<b>-Tet</b>	14.08	19.03	17.93	8.89	13.60	29.62
<b>Ratio</b>	<b>3.86</b>	<b>2.1</b>	<b>1.3</b>	<b>3.22</b>	<b>1.56</b>	<b>1.89</b>

The three ratios from each of the four experiments were then subjected to a One Sample t-test. This test assumes a normal sample distribution and tests the theory that the mean of the ratio is greater than a hypothesized mean of 1; i.e., the ratio would be 1 as the +Tet and –Tet samples would have equal volumes. The results of the test are shown in Table VII.3

**Table VII.3**

**Results of One Sample t-test**

**Hypothesized Mean = 1**

		<b>Mean</b>	<b>DF</b>	<b>t-value</b>	<b>P-value</b>
whole cell extracts	FL Shc	2.420	2	1.878	0.2012
	Y317F	2.223	2	2.411	0.1374
Grb2SH2-GST pptn's	FL	2.883	2	2.684	0.1153
	Y317F	1.763	2	2.590	0.1223

The results demonstrate that even though the mean values indicate that there is generally a 2 fold decrease in endogenous p52<sup>Shc</sup> tyrosine phosphorylation upon expression of FL Shc and Y317F Shc, technically, the results are not statistically significant (p value not less than 0.05). Expression of FL Shc resulted in a mean 2.420 fold decrease in endogenous p52<sup>Shc</sup> phosphorylation in whole cell extracts after IL-3 stimulation for 10 minutes. Similarly, expression of the Y317F Shc mutant resulted in a mean 2.223 fold decrease in endogenous p52<sup>Shc</sup> tyrosine phosphorylation. Biochemically, these changes appear significant but when subjected to statistical analysis, are proved not to be significant. This has a lot to do with the

number of experiments performed and if another one of two experiments were performed, bringing the total number of experiments to 4 or 5, the results would most likely be statistically significant. Similar results was also observed in experiments where the fold decrease in tyrosine phosphorylation endogenous p52<sup>Shc</sup> precipitated by Grb2SH2-GST was determined in cells expressing FL and Y317F Shc. Expression of FL Shc and Y317F Shc resulted in a mean 2.883 and 1.763 fold decrease in tyrosine phosphorylated p52<sup>Shc</sup> precipitated by the Grb2SH2-GST fusion protein, but again the One Sample t-test found this to not be statistically significant.

## References

- Adachi, M., Sekiya, M., Isobe, M., Kumura, Y., Ogita, Z., Hinoda, Y., Imai, K. and Yachi, A. (1992) Molecular-cloning and chromosomal mapping of a human protein-tyrosine phosphatase ptp1c. *Biochem. Biophys. Res. Comm.* **186**, 1607-1615.
- Adachi, T., Pazdrak, K., Stafford, S. and Alam, R. (1999) The mapping of the Lyn kinase binding site of the common beta subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor. *J. Immunol.* **162**, 1496-1501.
- Ahmad, S., Banville, D., Zhao, Z.Z., Fischer, E.H. and Shen, S.H. (1993) A widely expressed human protein-tyrosine phosphatase containing src homology-2 domains. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2197-2201.
- Alessi, D.R., Smythe, C. and Keyse, S.M. (1993) The human CL100 gene encodes a tyr/thr-protein phosphatase which potently and specifically inactivates map kinase and suppresses its activation by oncogenic ras in xenopus-oocyte extracts. *Oncogene* **8**, 2015-2020.
- Alessi, D.R., Saito, Y., Campbell, D.G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C.J. and Cowley, S. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by p74<sup>raf-1</sup>. *EMBO J.* **13**, 1610-1619.
- Ali, S., Chen, Z., Lebrun, J.J., Vogel, W., Kharitonov, A., Kelly, P.A. and Ullrich, A. (1996) PTP1D is a positive regulator of the prolactin signal leading to  $\beta$ -casein promoter activation. *EMBO J.* **15**, 135-142.
- Anderson, N.G., Ping, L.I., Marsden, L.A., Williams, N., Roberts, T.M. and Sturgill, T.W. (1991) Raf-1 is a potential substrate for mitogen-activated protein-kinase in vivo. *Biochem. J.* **277**, 573-576.
- Anderson, S.M. and Jorgensen, B. (1995) Activation of src-related tyrosine kinases by IL-3. *J. Immunol.* **155**, 1660-1670.
- Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N., Yokota, T. (1990) Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* **59**, 783-836.
- Argetsinger, L.S., Campbell, G.S., Yang, X.N., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N. and Coppers, C. (1993) Identification of Jak2 as a growth-hormone receptor-associated tyrosine kinase. *Cell* **74**, 237-244.
- Aronheim, A., Engelberg, D., Li, N.X., Alalawi, N., Schlessinger, J. and Karin, M. (1994) Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell* **78**, 949-961.



- Arrandale, J.M., GoreWillse, A., Rocks, S., Ren, J.M., Zhu, J., Davis, A., Livingston, J.N. and Rabin, D.U. (1996) Insulin signaling in mice expressing reduced levels of Syp. *J. Biol. Chem.* **271**, 21353-21358.
- Azam, M., Erdjumentbromage, H., Kreider, B.L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J.N. and Schindler, C. (1995) Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J.* **14**, 1402-1411.
- Azoulay, M., Webb, C.G. and Sachs, L. (1987) Control of hematopoietic-cell growth-regulators during mouse fetal development. *Mol. Cell. Biol.* **7**, 3361-3364.
- Bagley, C.J., Woodcock, J.M., Stomski, F.C. and Lopez, A.F. (1997) The structural and functional basis of cytokine receptor activation: lessons from the common beta subunit of the granulocyte-macrophage colony-stimulating factor, Interleukin-3 (IL-3), and IL-5 receptors. *Blood* **89**, 1471-1482.
- Barbacid, M. (1987) Ras genes. *Annu. Rev. Biochem.* **56**, 779-827.
- Barford, D., Jia, Z.C. and Tonks, N.K. (1995) Protein-tyrosine phosphatases take-off. *Nature Struc. Biol.* **2**, 1043-1053.
- Bazan, J.F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934-6938.
- Beck, T.W., Huleihel, M., Gunnell, M., Bonner, T.I. and Rapp, U.R. (1987) The complete coding sequence of the human A-Raf-1 oncogene and transforming activity of a human A-Raf carrying retrovirus. *Nucleic Acids Res.* **15**, 595-609.
- Bennett, A.M., Tang, T.L., Sugimoto, S., Walsh, C.T. and Neel, B.G. (1994) Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor  $\beta$  to Ras. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7335-7339.
- Bennett, A.M., Hausdorff, S.F., O'Reilly, A.M., Freeman, A.S. and Neel, B.G. (1996) Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression. *Mol. Cell. Biol.* **16**, 1189-1202.
- Berg, J.M. (1986) Potential metal-binding domains in nucleic-acid binding-proteins. *Science* **232**, 485-487.
- Blaikie, P., Immanuel, D., Wu, J., Li, N.X., Yajnik, V. and Margolis, B. (1994) A region in Shc distinct from the SH2 domain can bind tyrosine- phosphorylated growth-factor receptors. *J. Biol. Chem.* **269**, 32031-32034.
- Blaikie, P.A., Fournier, E., Dilworth, S.M., Birnbaum, D., Borg, J.-P. and Margolis, B. (1997) The role of the Shc phosphotyrosine interaction/phosphotyrosine binding domain and tyrosine phosphorylation sites in polyoma middle T antigen-mediated cell transformation. *J. Biol. Chem.* **272**, 20671-20677.

- Bone, H., Dechert, U., Jirik, F., Schrader, J.W. and Welham, M.J. (1997) SHP1 and SHP2 protein-tyrosine phosphatases associate with  $\beta c$  after interleukin-3-induced receptor tyrosine phosphorylation. *J.Biol.Chem.* **272**,
- Bonfini, L., Karlovich, C.A., Dasgupta, C. and Banerjee, U. (1992) The son of sevenless gene-product - a putative activator of Ras. *Science* **255**, 603-606.
- Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L. and Pelicci, P. (1996) Not all Shc's roads lead to Ras. *Trends Biochem. Sci.* **21**, 257-261.
- Borrello, M.G., Pelicci, G., Arighi, E., Defilippis, L., Greco, A., Bongarzone, I., Rizzetti, M.G., Pelicci, P.G. and Pierotti, M.A. (1994) The oncogenic versions of the ret and trk tyrosine kinases bind shc and grb2 adapter proteins. *Oncogene* **9**, 1661-1668.
- Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., Depinho, R.A., Panayotatos, N., Cobb, M.H. and Yancopoulos, G.D. (1991) Erks - a family of protein-serine threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**, 663-675.
- Boulton, T.G., Stahl, N. and Yancopoulos, G.D. (1994) Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J. Biol. Chem.* **269**, 11648-11655.
- Bowtell, D., Fu, P., Simon, M. and Senior, P. (1992) Identification of murine homologues of the *Drosophila* son of sevenless gene - potential activators of ras. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6511-6515.
- Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilising the principle of protein-dye. *Anal. Biochem.* **72**, 248-255.
- Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) The *saccharomyces-cerevisiae* cdc25 gene-product regulates the ras/adenylate cyclase pathway. *Cell* **48**, 789-799.
- Brondello, J.M., Brunet, A., Pouyssegur, J. and McKenzie, F.R. (1997) The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44(MAPK) cascade. *J. Biol. Chem.* **272**, 1368-1376.
- Bruder, J.T., Heidecker, G. and Rapp, U.R. (1992) Serum-induced, TPA-induced, and ras-induced expression from AP-1/ets- driven promoters requires raf-1 kinase. *Genes and Dev.* **6**, 545-556.
- Brunet, A., Pages, G. and Pouyssegur, J. (1994) Growth factor-stimulated map kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (Mek1). *FEBS Lett.* **346**, 299-303.

- Buday, L. and Downward, J. (1993a) Epidermal growth-factor regulates p21(Ras) through the formation of a complex of receptor, grb2 adapter protein, and sos nucleotide exchange factor. *Cell* **73**, 611-620.
- Buday, L. and Downward, J. (1993b) Epidermal growth-factor regulates the exchange-rate of guanine- nucleotides on p21ras in fibroblasts. *Mol. Cell. Biol.* **13**, 1903-1910.
- Burd, P.R., Rogers, H.W., Gordon, J.R., Martin, C.A., Jayaraman, S., Wilson, D.S., Dvorak, A.M., Galli, S.J. and Dorf, M.E. (1989) Interleukin-3-dependent and interleukin-3-independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* **170**, 245-257.
- Burgering, B.M.T., Medema, R.H., Maassen, J.A., Vandewetering, M.L., Vandereb, A.J., McCormick, F. and Bos, J.L. (1991) Insulin stimulation of gene-expression mediated by p21ras activation. *EMBO J.* **10**, 1103-1109.
- Burgering, B.M.T. and Coffey, P.J. (1995) Protein-Kinase-B (C-Akt) in phosphatidylinositol-3-OH kinase signal- transduction. *Nature* **376**, 599-602.
- Burshtyn, D.N., Scharenberg, A.M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T.L., Kinet, J.P. and Long, E.O. (1996) Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. *Immunity* **4**, 77-85.
- Burton, E.A., Hunter, S., Wu, S.C. and Anderson, S.M. (1997) Binding of src-like kinases to the beta-subunit of the interleukin-3 receptor. *J. Bio. Chem.* **272**, 16189-16195.
- Buss, J.E. and Sefton, B.M. (1986) Direct identification of palmitic acid as the lipid attached to p21Ras. *Mol. Cell. Biol.* **6**, 116-122.
- Cai, H., Szeberenyi, J. and Cooper, G.M. (1990) Effect of a dominant inhibitory Ha-Ras mutation on mitogenic signal transduction in NIH 3T3 cells. *Mol. Cell. Biol.* **10**, 5314-5323.
- Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* **64**, 281-302.
- Carlberg, K. and Rohrschneider, L.R. (1997) Characterization of a novel tyrosine phosphorylated 100-kDa protein that binds to SHP-2 and phosphatidylinositol 3'-Kinase in myeloid cells. *J. Biol. Chem.* **272**, 15943-15950.
- Case, R.D., Piccione, E., Wolf, G., Benett, A.M., Lechleider, R.J., Neel, B.G. and Shoelson, S.E. (1994) SH-PTP2/Syp SH2 domain binding specificity is defined by direct interactions with platelet-derived growth factor  $\beta$ -receptor, epidermal growth factor receptor, and insulin receptor substrate-1-derived phosphopeptides. *J. Biol. Chem.* **269**, 10467-10474.

- Catling, A.D., Schaeffer, H.J., Reuter, C.W.M., Reddy, G.R. and Weber, M.J. (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. *Mol. Cell. Biol.* **15**, 5214-5225.
- Chardin, P., Camonis, J.H., Gale, N.W., Vanaelst, L., Schlessinger, J., Wigler, M.H. and Barsagi, D. (1993) Human *sos1* - a guanine-nucleotide exchange factor for ras that binds to *grb2*. *Science* **260**, 1338-1343.
- Charles, C.H., Abler, A.S. and Lau, L.F. (1992) cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. *Oncogene* **7**, 187-190.
- Chen, H.E., Chang, S., Trub, T. and Neel, B.G. (1996) Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol. Cell. Biol.* **16**, 3685-3697.
- Cherniack, A.D., Klarlund, J.K. and Czech, M.P. (1994) Phosphorylation of the ras nucleotide exchange factor son of sevenless by mitogen-activated protein-kinase. *J. Biol. Chem.* **269**, 4717-4720.
- Clark-Lewis, I., Kent, S.B.H. and Schrader, J.W. (1984) Purification to apparent homogeneity of a factor stimulating the growth of multiple lineages of hematopoietic-cells. *J. Biol. Chem.* **259**, 7488-7494.
- Clark-Lewis, I., Aebersold, R., Ziltener, H.J., Schrader, J.W., Hood, L.E. and Kent, S.B.H. (1986) Automated chemical synthesis of a protein-growth factor for hematopoietic-cells, interleukin-3. *Science* **231**, 134-139.
- Clark, S.G., Stern, M.J. and Horvitz, H.R. (1992) C-elegans cell-signaling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.
- Cohen, G.B., Ren, R.B. and Baltimore, D. (1995) Modular binding domains in signal-transduction proteins. *Cell* **80**, 237-248.
- Colosi, P., Wong, K., Leong, S.R. and Wood, W.I. (1993) Mutational analysis of the intracellular domain of the human growth- hormone receptor. *J. Biol. Chem.* **268**, 12617-12623.
- Corbalan-Garcia, S., Yang, S.S., Degenhardt, K.R. and Barsagi, D. (1996) Identification of the mitogen-activated protein kinase phosphorylation sites on human *Sos1* that regulate interaction with *Grb2*. *Mol. Cell. Biol.* **16**, 5674-5682.
- Craddock, B.L. and Welham, M.J. (1997) Interleukin-3 induces association of the protein-tyrosine phosphatase SHP2 and phosphatidylinositol 3-kinase with a 100-kDa tyrosine-phosphorylated protein in hemopoietic cells. *J. Biol. Chem.* **272**, 29281-29289.

- Craddock, B.L., Orchiston, E.A., Hinton, H.J. and Welham, M.J. (1999) Expression of dominant negative phosphoinositide-3-kinase leads to a decrease in IL-3-dependent proliferation and PKB activation, with only minimal effects on apoptosis. *J. Biol. Chem.* **274**, 10633-10640.
- Crapper, R.M., Thomas, W.R. and Schrader, J.W. (1984) In vivo transfer of persisting (P) cells-further evidence for their identity with T-dependent mast-cells. *J. Immunol.* **133**, 2174-2179.
- Crews, C.M., Alessandrini, A. and Erikson, R.L. (1992) The primary structure of MEK, a protein-kinase that phosphorylates the erk gene-product. *Science* **258**, 478-480.
- Cross, D.A.E., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S. and Cohen, P. (1994) The inhibition of glycogen-synthase kinase-3 by insulin or insulin-like growth-factor-1 in the rat skeletal-muscle cell-line-l6 is blocked by wortmannin, but not by rapamycin - evidence that wortmannin blocks activation of the mitogen-activated protein-kinase pathway in l6-cells between ras and raf. *Biochem. J.* **303**, 21-26.
- Cutler, R.L., Liu, L., Damen, J.E. and Krystal, G. (1993) Multiple cytokines induce the tyrosine phosphorylation of shc and its association with grb2 in hematopoietic-cells. *J. Biol. Chem.* **268**, 21463-21465.
- D'Ambrosio, D., Hippen, K.L., Minskoff, S.A., Mellman, I., Pani, G., Siminovitch, K.A. and Cambier, J.C. (1995) Recruitment and activation of ptp1c in negative regulation of antigen receptor signaling by Fcγ-RIIB1. *Science* **268**, 293-297.
- Damen, J.E., Liu, L., Cutler, R.L. and Krystal, G. (1993) Erythropoietin stimulates the tyrosine phosphorylation of shc and its association with grb2 and a 145-kd tyrosine-phosphorylated protein. *Blood* **82**, 2296-2303.
- Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W. and Krystal, G. (1996) The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1689-1693.
- Damen, J.E., Liu, L., Ware, M.D., Ermolaeva, M., Majerus, P.W. and Krystal, G. (1998) Multiple forms of the SH2-containing inositol phosphatase, SHIP, are generated by C-terminal truncation. *Blood* **92**, 1199-1205.
- Darnell, J.E., Kerr, I.M. and Stark, G.R. (1994) Jak-stat pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-1421.
- Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H.A., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241.

- David, M., Chen, H.Y.E., Goelz, S., Lerner, A.C. and Neel, B.G. (1995) Differential regulation of the alpha/beta interferon-stimulated jak/stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol. Cell. Biol.* **15**, 7050-7058.
- Dechert, U., Adam, M., Harder, K.W., Clark-Lewis, I., and Jirik, F. (1994) Comparison of the protein tyrosine phosphatase SH-PTP2: Study of phosphopeptide substrate and possible regulatory role for SH2 domains. *Eur. J. Biochem.* **231**, 673-681.
- Dechert, U., Affolter, M., Harder, K.W., Matthews, J., Owen, P., Clark-Lewis, I., Thomas, M.L., Aebersold, R. and Jirik, F.R. (1995) Comparison of the specificity of bacterially expressed cytoplasmic protein-tyrosine phosphatases SHP and SH-PTP2 towards synthetic phosphopeptide substrates. *Eur. J. Biochem.* **231**, 673-681.
- Del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**, 687-689.
- Dent, P., Campbell, D.G., Caudwell, F.B. and Cohen, P. (1990) Identification of 3 in vivo phosphorylation sites on the glycogen-binding subunit of protein phosphatase-1 from rabbit skeletal-muscle, and their response to adrenaline. *FEBS Lett.* **259**, 281-285.
- Dent, P., Haser, W., Haystead, T.A.J., Vincent, L.A., Roberts, T.M. and Sturgill, T.W. (1992) Activation of mitogen-activated protein-kinase kinase by v-raf in NIH 3T3 cells and in vitro. *Science* **257**, 1404-1407.
- Devos, R., Plaetinck, G., Vanderheyden, J., Cornelis, S., Vandekerckhove, J., Fiers, W. and Tavernier, J. (1991) Molecular-basis of a high-affinity murine interleukin-5 receptor. *EMBO J.* **10**, 2133-2137.
- Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M.J., Yonezawa, K., Kasuga, M. and Waterfield, M.D. (1994) PI3-kinase-structure and functional analysis of intersubunit interactions. *EMBO J.* **13**, 511-521.
- Dikic, I., Batzer, A.G., Blaikie, P., Obermeier, A., Ullrich, A., Schlessinger, J. and Margolis, B. (1995) Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. *J. Biol. Chem.* **270**, 15125-15129.
- Dilworth, S.M., Brewster, C.E.P., Jones, M.D., Lanfranccone, L., Pelicci, G. and Pelicci, P.G. (1994) Transformation by polyoma-virus middle t-antigen involves the binding and tyrosine phosphorylation of shc. *Nature* **367**, 87-90.
- Doody, G.M., Justement, L.B., Delibrias, C.C., Matthews, R.J., Lin, J., Thomas, M.L. and Fearon, D.T. (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science* **269**, 242-244.
- Dorsch, M., Hock, H. and Diamantstein, T. (1994) Tyrosine phosphorylation of Shc is induced by IL-3, IL-5 and GM-CSF. *Biochem. Biophys. Res. Comm.* **200**, 562-568.

- Downward, J., Graves, J.D., Warne, P.H., Rayter, S. and Cantrell, D.A. (1990) Stimulation of p21ras upon T-cell activation. *Nature* **346**, 719-723.
- Duronio, V., Clark-Lewis, I., Federspiel, B., Wieler, J.S. and Schrader, J.W. (1992a) Tyrosine phosphorylation of receptor beta subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. *J. Biol. Chem.* **267**, 21856-21863.
- Duronio, V., Welham, M.J., Abraham, S., Dryden, P. and Schrader, J.W. (1992b) p21<sup>ras</sup> activation via hemopoietin receptors and c-kit requires tyrosine kinase activity but not tyrosine phosphorylation of p21<sup>ras</sup> GTPase-activating protein. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1587-1591.
- Durstin, M., Inhorn, R.C. and Griffin, J.D. (1996) Tyrosine phosphorylation of Shc is not required for proliferation or viability signaling by granulocyte-macrophage colony-stimulating factor in hematopoietic cell lines. *J. Immunol.* **157**, 534-540.
- Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. and Weinberg, R.A. (1993) Association of Sos ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**, 45-51.
- Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal-antibodies specific for human c-Myc proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610-3616.
- Eychene, A., Barnier, J.V., Dezelee, P., Marx, M., Laugier, D., Calogeraki, I. and Calothy, G. (1992) Quail neuroretina c-rnil (B-raf) protooncogene cDNAs encode 2 proteins of 93.5-Kda and 95-kda resulting from alternative splicing. *Oncogene* **7**, 1315-1323.
- Fabian, J.R., Daar, I.O. and Morrison, D.K. (1993) Critical tyrosine residues regulate the enzymatic and biological- activity of Raf-1 kinase. *Mol. Cell. Biol.* **13**, 7170-7179.
- Feig, L.A. and Cooper, G.M. (1988) Inhibition of NIH-3T3 cell-proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.* **8**, 3235-3243.
- Feig, L.A. (1993) The many roads that lead to ras. *Science* **260**, 767-768.
- Feng, G.S., Hui, C.C. and Pawson, T. (1993) SH2-containing phosphotyrosine phosphatase as a target of protein- tyrosine kinases. *Science* **259**, 1607-1611.
- Ferby, I.M., Waga, I., Sakanaka, C., Kume, K. and Shimizu, T. (1994) Wortmannin inhibits mitogen-activated protein-kinase activation- induced by platelet-activating-factor in guinea-pig neutrophils. *J. Biol. Chem.* **269**, 30485-30488.
- Firmbach-Kraft, I., Byers, M., Shows, T., Dallafavera, R. and Krolewski, J.J. (1990) Tyk2, prototype of a novel class of nonreceptor tyrosine kinase genes. *Oncogene* **5**, 1329-1336.

- Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R. and Tsichlis, P.N. (1995) The protein-kinase encoded by the akt protooncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727-736.
- Freeman, R., Plutsky, J. and Neel, B.G. (1992) Identification of a human src homology 2-containing protein-tyrosine-phosphatase: A putative homolog of *Drosophila* corkscrew. *Proc. Natl. Acad. Sci.* **89**, 11239-11243.
- Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T. and Kasuga, M. (1996) A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol.* **16**, 6887-6899.
- Fung, M.C., Hapel, A.J., Ymer, S., Cohen, D.R., Johnson, R.M., Campbell, H.D. and Young, I.G. (1984) Molecular-cloning of cDNA for murine interleukin-3. *Nature* **307**, 233-237.
- Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J. and Barsagi, D. (1993) Grb2 mediates the EGF-dependent activation of guanine-nucleotide exchange on ras. *Nature* **363**, 88-92.
- Giordano, V., DeFalco, G., Chiari, R., Quinto, I., Pelicci, P.G., Bartholomew, L., Delmastro, P., Gadina, M. and Scala, G. (1997) Shc mediates IL-6 signaling by interacting with gp130 and Jak2 kinase. *J. Immunol.* **158**, 4097-4103.
- Gold, M.R., Duronio, V., Saxena, S.P., Schrader, J.W. and Aebersold, R. (1994) Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells. Association of the enzyme with various tyrosine-phosphorylated proteins. *J. Biol. Chem.* **269**, 5403-5412.
- Gonzalez, F.A., Raden, D.L. and Davis, R.J. (1991) Identification of substrate recognition determinants for human erk1 and erk2 protein-kinases. *J. Biol. Chem.* **266**, 22159-22163.
- Gorman, D.M., Itoh, N., Kitamura, T., Schreurs, J., Yonehara, S., Yahara, I., Arai, K.I. and Miyajima, A. (1990) Cloning and expression of a gene encoding an interleukin-3 receptor-like protein - identification of another member of the cytokine receptor gene family. *Proc. Natl. Acad. Sci.* **87**, 5459-5463.
- Gossen, M. and Bujard, H. (1992) Tight control of gene-expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547-5551.
- Gossen, M., Bonin, A.L. and Bujard, H. (1993) Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem. Sci.* **18**, 471-475.
- Gotoh, N., Tojo, A., Muroya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y. and Shibuya, M. (1994) Epidermal growth factor-receptor mutant lacking



- the autophosphorylation sites induces phosphorylation of shc protein and shc-grb2 association and retains mitogenic activity. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 167-171.
- Gotoh, N., Muroya, K., Hattori, S., Nakamura, S., Chida, K. and Shibuya, M. (1995) The SH2 domain of Shc suppresses EGF-induced mitogenesis in a dominant negative manner. *Oncogene* **11**, 2525-2533.
- Gotoh, N., Tojo, A. and Shibuya, M. (1996) A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3. *EMBO J.* **15**, 6197-6204.
- Gotoh, N., Toyoda, M. and Shibuya, M. (1997) Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation. *Mol. Cell. Biol.* **17**, 1824-1831.
- Grammer, T.C. and Blenis, J. (1997) Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. *Oncogene* **14**, 1635-1642.
- Gu, H., Pratt, J.C., Burakoff, S.J. and Neel, B.G. (1998) Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Molecular Cell* **2**, 729-740.
- Gualtieri, R.J., Liang, C.M., Shadduck, R.K., Waheed, A. and Banks, J. (1987) Identification of the hematopoietic growth factors elaborated by bone-marrow stromal cells using antibody neutralization analysis. *Exp. Hematol.* **15**, 883-889.
- Gustafson, T.A., He, W.M., Craparo, A., Schaub, C.D. and Oneill, T.J. (1995) Phosphotyrosine-dependent interaction of shc and IRS-1 with the NPEY motif of the insulin-receptor via a novel (non-SH2) domain. *Diabetes* **44**, A49
- Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.
- Hapel, A.J., Lee, J.C., Farrar, W.L. and Ihle, J.N. (1981) Establishment of continuous cultures of THY1.2<sup>+</sup>, LYT1<sup>+</sup>, 2-T-cells with purified interleukin-3. *Cell* **25**, 179-186.
- Hapel, A.J. and Young, I.G. (1988) In: *Lymphokines 15; Interleukin 3: The panspecific hemopoietin* (ed. J.W. Schrader), Academic Press, San Diego, CA, p91-126.
- Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T.R., Hawkins, P.T., Dhand, R., Clark, A.E., Holman, G.D., Waterfield, M.D. and Kasuga, M. (1994) 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose-transport but not for ras activation in CHO cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7415-7419.

- Hara, T. and Miyajima, A. (1992) 2 Distinct functional high-affinity receptors for mouse interleukin-3 (IL-3). *EMBO J.* **11**, 1875-1884.
- Harder, K.W., Owen, P., Wong, L.K.H., Aebersold, R., Clark-Lewis, I., and Jirik, F.R. (1994) Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase  $\beta$  (HPTP $\beta$ ) using synthetic phosphopeptides. *Mol. Cell. Biol.* **298**, 395-401.
- Harvey, J.J. (1964) An unidentified virus which causes the rapid production of tumours in mice. *Nature* **204**, 1104-1105.
- Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K.I., Yokota, T. and Miyajima, A. (1990) Molecular-cloning of a 2nd subunit of the receptor for human granulocyte macrophage colony-stimulating factor (GM-CSF) - Reconstitution of a high-affinity GM-CSF receptor. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9655-9659.
- Heidecker, G., Huleihel, M., Cleveland, J.L., Kolch, W., Beck, T.W., Lloyd, P., Pawson, T. and Rapp, U.R. (1990) Mutational activation of c-raf-1 and definition of the minimal transforming sequence. *Mol. Cell. Biol.* **10**, 2503-2512.
- Holt, K.H., Olson, A.L., Moyerowley, W.S. and Pessin, J.E. (1994) Phosphatidylinositol 3-kinase activation is mediated by high-affinity interactions between distinct domains within the p110 and p85 subunits. *Mol. Cell. Biol.* **14**, 42-49.
- Howe, L.R., Leever, S.J., Gomez, N., Nakielnny, S., Cohen, P. and Marshall, C.J. (1992) Activation of the map kinase pathway by the protein-kinase raf. *Cell* **71**, 335-342.
- Hu, Q.J., Klippel, A., Muslin, A.J., Fantl, W.J. and Williams, L.T. (1995) Ras-dependent induction of cellular-responses by constitutively active phosphatidylinositol-3 kinase. *Science* **268**, 100-102.
- Huyer, G., Li, Z.M., Adam, M., Huckle, W.R. and Ramachandran, C. (1995) Direct determination of the sequence recognition requirements of the SH2 domains of SH-PTP2. *Biochemistry* **34**, 1040-1049.
- Ihle, J.N., Pepersack, L. and Rebar, L. (1981) Regulation of T-cell differentiation-in vitro induction of 20-alpha-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. *J. Immunol.* **126**, 2184-2189.
- Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T.D., Fitch, F., Prystowsky, M.B., Goldwasser, E., Schrader, J.W., Palaszynski, E., Dy, M. and Lebel, B. (1983) Biologic properties of homogenous interleukin-2. *J. Immunol.* **131**, 282-287.
- Ihle, J.N. (1992) Interleukin-3 and hematopoiesis. *Chem. Immunol.* **51**, 65-106.
- Ihle, J.N. (1996) STATs: Signal transducers and activators of transcription. *Cell* **84**, 331-334.

- Inhorn, R.C., Carlesso, N., Durstin, M., Frank, D.A. and Griffin, J.D. (1995) Identification of a viability domain in the granulocyte/macrophage colony-stimulating factor receptor beta-chain involving tyrosine-750. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8665-8669.
- Ip, Y.T. and Davis, R.J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. *Curr. Opin. Cell. Biol.* **10**, 205-219.
- Itoh, N., Yonehara, S., Schreurs, J., Gorman, D.M., Maruyama, K., Ishii, A., Yahara, I., Arai, K. and Miyajima, A. (1990) Cloning of an interleukin-3 receptor gene - a member of a distinct receptor gene family. *Science* **247**, 324-327.
- Itoh, T., Muto, A., Watanabe, S., Miyajima, A., Yokota, T. and Arai, K.I. (1996) Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of c-fos through different modes of signaling. *J. Biol. Chem.* **271**, 7587-7592.
- Jascur, T., Gilman, J. and Mustelin, T. (1997) Involvement of phosphatidylinositol 3-kinase in NFAT activation in T cells. *J. Biol. Chem.* **272**, 14483-14488.
- Jelinek, T., Catling, A.D., Reuter, C.W.M., Moodie, S.A., Wolfman, A. and Weber, M.J. (1984) RAS and RAF-1 form a signalling complex with MEK-1 but not MEK-2. *Mol. Cell. Biol.* **14**, 8212-8218.
- Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platanias, L.C. and Yi, T. (1996) Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol. Cell. Biol.* **16**, 6985-6992.
- Jiao, H.Y., Yang, W.T., Berrada, K., Tabrizi, M., Shultz, L. and Yi, T.L. (1997) Macrophages from motheaten and viable motheaten mutant mice show increased proliferative responses to GM-CSF: Detection of potential HCP substrates in GM-CSF signal transduction. *Exp. Hematol.* **25**, 592-600.
- Kallunki, T., Deng, T.L., Hibi, M. and Karin, M. (1996) c-jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* **87**, 929-939.
- Kauffman-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffey, P., Downward, J. and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-548.
- Kavanaugh, W.M. and Williams, L.T. (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* **266**, 1862-1865.
- Kavanaugh, W.M., Truck, C.W. and Williams, L.T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268**, 1177-1179.

- Kavanaugh, W.M., Pot, D.A., Chin, S.M., Deutereinhard, M., Jefferson, A.B., Norris, F.A., Masiarz, F.R., Cousens, L.S., Majerus, P.W. and Williams, L.T. (1996) Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and Grb2. *Current Biol.* **6**, 438-445.
- Kazlauskas, A., Feng, G.S., Pawson, T. and Valius, M. (1993) The 64-kDa protein that associates with the platelet-derived growth factor receptor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6939-6943.
- Keyse, S.M. and Emslie, E.A. (1992) Oxidative stress and heat-shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature* **359**, 644-647.
- Kharitonov, A., Chen, Z., Sures, I., Wang, H., Schilling, J. and Ullrich, A. (1997) A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature* **386**, 181-186.
- Kinoshita, T., Yokota, T., Arai, K. and Miyajima, A. (1995) Suppression of apoptotic death in hematopoietic-cells by signaling through the IL-3/GM-CSF receptors. *EMBO J.* **14**, 266-275.
- Kirsten, W.H. and Mayer, L.A. (1967) Morphological responses to a murine erythroblastosis virus. *J. Natl. Can. Inst.* **39**, 311-335.
- Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.-F., Miyazono, K., Urabe, A., and Takaku, F. (1989) Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or Erythropoietin. *J. Cell. Physiol.* **140**, 323-334.
- Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K. and Miyajima, A. (1991) Reconstitution of functional receptors for human granulocyte macrophage colony-stimulating factor (GM-CSF) - Evidence that the protein encoded by the Aic2B cDNA is a subunit of the murine GM-CSF receptor. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5082-5086.
- Klinghoffer, R.A. and Kazlauskas, A. (1995) Identification of a putative Syp substrate, the PDGF beta receptor. *J. Biol. Chem.* **270**, 22208-22217.
- Klinghoffer, R.A., Duckworth, B., Valius, M., Cantley, L. and Kazlauskas, A. (1996) Platelet-derived growth factor-dependent activation of phosphatidylinositol 3-kinase is regulated by receptor binding of SH2- domain-containing proteins which influence Ras activity. *Mol. Cell. Biol.* **16**, 5905-5914.
- Klingmuller, U., Lorenz, U., Cantley, L.C., Neel, B.G. and Lodish, H.F. (1995) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* **80**, 729-738.

- Klippel, A., Escobedo, J.A., Hirano, M. and Williams, L.T. (1994) The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme-activity. *Mol. Cell. Biol.* **14**, 2675-2685.
- Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A. and Williams, L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* **16**, 4117-4127.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 domains - elements that control interactions of cytoplasmic signaling proteins. *Science* **252**, 668-674.
- Kodaki, T., Woscholski, R., Hallberg, B., Rodriguezviciano, P., Downward, L. and Parker, P.J. (1994) The activation of phosphatidylinositol 3-kinase by ras. *Current Biol.* **4**, 798-806.
- Kolch, W., Heidecker, G., Lloyd, P. and Rapp, U.R. (1991) Raf-1 protein-kinase is required for growth of induced NIH/3T3 cells. *Nature* **349**, 426-428.
- Kuhne, M.R., Pawson, T., Lienhard, G.E. and Feng, G.S. (1993) The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. *J. Biol. Chem.* **268**, 11479-11481.
- Kyriakis, J.M., App, H., Zhang, X.F., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1992) Raf-1 activates map kinase-kinase. *Nature* **358**, 417-421.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227**, 680-684.
- Lai, K.M.V., Olivier, J.P., Gish, G.D., Henkemeyer, M., McGlade, J. and Pawson, T. (1995) A Drosophila shc gene-product is implicated in signaling by the DER receptor tyrosine kinase. *Mol. Cell. Biol.* **15**, 4810-4818.
- Laminet, A.A., Apell, G., Conroy, L. and Kavanaugh, W.M. (1996) Affinity, specificity, and kinetics of the interaction of the Shc phosphotyrosine binding domain with asparagine-X-X-phosphotyrosine motifs of growth-factor receptors. *J. Biol. Chem.* **271**, 264-269.
- Lamkin, T.D., Walk, S.F., Liu, L., Damen, J.E., Krystal, G. and Ravichandran, S. (1997) Shc interaction with Scr homology 2 domain containing inositol phosphatase (SHIP) in vivo requires Shc-phosphotyrosine binding domain and two specific phosphotyrosines on SHIP. *J. Biol. Chem.* **272**, 10396-10401.
- Lane, H.A., Fernandez, A., Lamb, N.J.C. and Thomas, G. (1993) p70(S6K) function is essential for G1-progression. *Nature* **363**, 170-172.
- Lanfrancone, L., Pelicci, G., Brizzi, M.F., Aronica, M.G., Casciari, C., Giuli, S., Pegoraro, L., Pawson, T. and Pelicci, P.G. (1995) Overexpression of shc proteins potentiates the

- proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of grb2/sos and grb2/p140 complexes to the  $\beta$ -receptor subunit. *Oncogene* **10**, 907-917.
- Lantz, C.S., Boesiger, J., Song, C.H., Mach, N., Kobayashi, T., Mulligan, R.C., Nawa, Y., Dranoff, G. and Galli, S.J. (1998) Role for interleukin-3 in mast cell and basophil development and immunity to parasites. *Nature* **392**, 90-93.
- Lechleider, R.J., Freeman, R. and Neel, B.G. (1993a) Tyrosyl phosphorylation and growth factor receptor association of the human corkscrew homologue, SH-PTP2. *J. Biol. Chem.* **268**, 13434-13438.
- Lechleider, R.J., Sugimoto, S., Bennett, A.M., Kashishian, A.S., Cooper, J.A., Shoelson, S.E., Walsh, C.T. and Neel, B.G. (1993b) Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor beta. *J. Biol. Chem.* **268**, 21478-21481.
- Lee, C.H., Kominos, D., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S.E. and Kuriyan, J. (1994) Crystal-structures of peptide complexes of the amino-terminal SH2 domain of the src tyrosine phosphatase. *Structure* **2**, 423-438.
- Lee, R.M., Cobb, M.H. and Blackshear, P.J. (1992) Evidence that extracellular signal-regulated kinases are the insulin-activated raf-1 kinase kinases. *J. Biol. Chem.* **267**, 1088-1092.
- Leevers, S.J., Paterson, H.F. and Marshall, C.J. (1994) Requirement for ras in raf activation is overcome by targeting raf to the plasma-membrane. *Nature* **369**, 411-414.
- Lenormand, P., Sardet, C., Pages, G., Lallemain, G., Brunet, A. and Pouyssegur, J. (1993) Growth-factors induce nuclear translocation of map kinases p42<sup>MAPK</sup> and p44<sup>MAPK</sup> but not of their activator map kinase kinase p45<sup>MAPKK</sup> in fibroblasts. *J. Cell. Biol.* **122**, 1079-1088.
- Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Barsagi, D., Margolis, B. and Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos1 binds to grb2 and links receptor tyrosine kinases to ras signaling. *Nature* **363**, 85-88.
- Li, S., Janosch, P., Tanji, M., Rosenfeld, G.C., Waymire, J.C., Mischak, H., Kolch, W. and Sedivy, J.M. (1995) Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. *EMBO J.* **14**, 685-696.
- Li, W., Nishimura, R., Kashishian, A., Batzer, A.G., Kim, W.J.H., Cooper, J.A. and Schlessinger, J. (1994) A new function for a phosphotyrosine phosphatase - linking grb2-sos to a receptor tyrosine kinase. *Mol. Cell. Biol.* **14**, 509-517.

- Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R.J. (1993) cPLA<sub>2</sub> is phosphorylated and activated by map kinase. *Cell* **72**, 269-278.
- Lioubin, M.N., Myles, G.M., Carlberg, K., Bowtell, D. and Rohrschneider, L.R. (1994) Shc, grb2, sos1, and a 150-kilodalton tyrosine-phosphorylated protein form complexes with fms in hematopoietic-cells. *Mol. Cell. Biol.* **14**, 5682-5691.
- Lioubin, M.N., Algate, P.A., Tsai, S., Carlberg, K., Aebersold, R. and Rohrschneider, L.R. (1996) p150(SHIP), a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes and Dev.* **10**, 1084-1095.
- Liu, L., Damen, J.E., Cutler, R.L. and Krystal, G. (1994) Multiple cytokines stimulate the binding of a common 145-kilodalton protein to shc at the grb2 recognition site of shc. *Mol. Cell. Biol.* **14**, 6926-6935.
- Liu, L., Damen, J.E., Hughes, M.R., Babic, I., Jirik, F.R. and Krystal, G. (1997a) The Src homology 2 (SH2) domain of SH2-containing inositol phosphatase (SHIP) is essential for tyrosine phosphorylation of SHIP, its association with Shc, and its induction of apoptosis. *J. Biol. Chem.* **272**, 8983-8988.
- Liu, L., Damen, J.E., Ware, M.D. and Krystal, G. (1997b) Interleukin-3 induces the association of the inositol 5-phosphatase SHIP with SHP2. *J. Biol. Chem.* **272**, 10998-11001.
- Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Barsagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain containing protein grb2 links receptor tyrosine kinases to ras signaling. *Cell* **70**, 431-442.
- Lowy, D.R. and Willumsen, B.M. (1993) Function and regulation of ras. *Ann. Rev. Biochem.* **62**, 851-891.
- Marais, R., Wynne, J. and Treisman, R. (1993) The srf accessory protein elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393.
- Marais, R., Light, Y., Paterson, H.F., and Marshall, C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.* **14**, 3136-3145.
- Marte, B.M., Rodriguezviciano, P., Wennstrom, S., Warne, P.H. and Downward, J. (1997) R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. *Current Biol.* **7**, 63-70.
- Matsuda, S., Gotoh, Y. and Nishida, E. (1993) Phosphorylation of xenopus mitogen-activated protein (Map) Kinase kinase by map kinase kinase kinase and map kinase. *J. Biol. Chem.* **268**, 3277-3281.

- Matsuguchi, T., Salgia, R., Hallek, M., Eder, M., Druker, B., Ernst, T.J. and Griffin, J.D. (1994) Shc phosphorylation in myeloid cells is regulated by granulocyte- macrophage colony-stimulating factor, interleukin-3, and steel factor and is constitutively increased by p210(Bcr/abl). *J. Biol. Chem.* **269**, 5016-5021.
- Matthews, R.J., Bowne, D.B., Flores, E. and Thomas, M.L. (1992) Characterization of hematopoietic intracellular protein tyrosine phosphatases - description of a phosphatase containing an SH2 domain and another enriched in proline-rich, glutamic acid-rich, serine- rich, and threonine-rich sequences. *Mol. Cell. Biol.* **12**, 2396-2405.
- McGlade, J., Cheng, A., Pelicci, G., Pelicci, P.G. and Pawson, T. (1992) Shc proteins are phosphorylated and regulated by the v-src and v-fps protein-tyrosine kinases. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8869-8873.
- Medema, R.H., Wubbolts, R. and Bos, J.L. (1991) 2 Dominant inhibitory mutants of p21ras interfere with insulin- induced gene-expression. *Mol. Cell. Biol.* **11**, 5963-5967.
- Metcalf, D. (1989) The molecular control of cell-division, differentiation commitment and maturation in hematopoietic-cells. *Nature* **339**, 27-30.
- Michaud, N.R.F.J.R., Mathes, K.D. and Morrison, D.K. (1995) 14-3-3 is not essential for Raf-1 function-identification of Raf-1 proteins that are biologically activated by 14-3-3-independent and Ras-independent manner. *Mol. Cell. Biol.* **15**, 3390-3397.
- Migliaccio, E., Mele, S., Salcini, A.E., Pelicci, G., Lai, K.M.V., SupertiFurga, G., Pawson, T., DiFiore, P., Lanfrancone, L. and Pelicci, P.G. (1997) Opposite effects of the p52(shc)/p46(shc) and p66(shc) splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. *EMBO J.* **16**, 706-716.
- Milarski, K.L. and Saltiel, A.R. (1994) Expression of catalytically inactive syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein-kinase by insulin. *J. Biol. Chem.* **269**, 21239-21243.
- Miura, O., Cleveland, J.L. and Ihle, J.N. (1993) Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. *Mol. Cell. Biol.* **13**, 1788-1795.
- Miyajima, A., Miyatake, S., Schreurs, J., Devries, J., Arai, N., Yokota, T. and Arai, K.I. (1988) Coordinate regulation of immune and inflammatory responses by T-cell-derived lymphokines. *FASEB J.* **2**, 2462-2473.
- Miyajima, A., Kitamura, T., Harada, N., Yokota, T. and Arai, K. (1992) Cytokine receptors and signal transduction. *Ann. Rev. Immunol.* **10**, 295-331.
- Morrison, D.K., Heidecker, G., Rapp, U.R. and Copeland, T.D. (1993) Identification of the major phosphorylation sites of the raf-1 kinase. *J. Biol. Chem.* **268**, 17309-17316.



- Mui, A.L.F., Wakao, H., Ofarrell, A.M., Harada, N. and Miyajima, A. (1995) Interleukin-3, Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-5 transduce signals through 2 Stat5 homologs. *EMBO J.* **14**, 1166-1175.
- Mui, A.L.F., Wakao, H., Kinoshita, T. and Miyajima, A. (1996) Suppression of interleukin-3-induced gene expression by a C-terminal truncated stat5: Role of stat5 in proliferation. *EMBO J.* **15**, 2425-2433.
- Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985) Requirement for ras proto-oncogene function during serum-stimulated growth of NIH-3T3-cells. *Nature* **313**, 241-243.
- Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. and Kishimoto, T. (1991) Critical cytoplasmic region of the interleukin-6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349-11353.
- Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84**, 889-897.
- Nakamura, T., Sanokawa, R., Sasaki, Y., Ayusawa, D., Oishi, M. and Mori, N. (1996) N-Shc: A neural-specific adapter molecule that mediates signaling from neurotrophin/Trk to Ras/MAPK pathway. *Oncogene* **13**, 1111-1121.
- Naparstek, E., Pierce, J., Metcalf, D., Shadduck, R.K., Ihle, J., Leder, A., Sakakeeny, M.A., Wagner, K., Falco, J., Fitzgerald, T.J. and Greenberger, S.J. (1986) Induction of growth alterations in factor-dependent hematopoietic progenitor-cell lines by cocultivation with irradiated bone-marrow stromal cells. *Blood* **67**, 1395-1403.
- Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T. and Taga, T. (1994) Activation of jak2 kinase mediated by the interleukin-6 signal transducer gp130. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2285-2289.
- Niemeyer, C.M., Sieff, C.A., Matheyprevot, B., Wimperis, J.Z., Bierer, B.E., Clark, S.C. and Nathan, D.G. (1989) Expression of human interleukin-3 (Multi-CSF) is restricted to human lymphocytes and T-cell tumor lines. *Blood* **73**, 945-951.
- Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y. and Kasuga, M. (1994) Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol. Cell. Biol.* **14**, 6674-6682.
- O'Bryan, J.P., Songyang, Z., Cantley, L.C., Der, C.J. and Pawson, T. (1996) A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2729-2734.

- O'Bryan, J.P., Lambert, Q.T. and Der, C.J. (1998) The src homology 2 and phosphotyrosine binding domains of the ShcC adaptor protein function as inhibitors of mitogenic signaling by the epidermal growth factor receptor. *J. Biol. Chem.* **273**, 20431-20437.
- Okuda, K., Smith, L., Griffin, J.D. and Foster, R. (1997) Signaling functions of the tyrosine residues in the  $\beta$ c chain of the granulocyte-macrophage colony-stimulating factor receptor. *Blood* **90**, 4759-4766.
- Ono, M., Bolland, S., Tempst, P. and Ravetch, J.V. (1996) Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc $\gamma$ -RIIB. *Nature* **383**, 263-266.
- Palacios, R., Henson, G., Steinmetz, M. and McKearn, J.P. (1984) Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. *Nature* **309**, 126-131.
- Palacios, R. and Steinmetz, M. (1985) IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate lymphocytes-B in vivo. *Cell* **41**, 727-734.
- Pani, G., Kozlowski, M., Cambier, J.C., Mills, G.B. and Siminovitch, K.A. (1995) Identification of the tyrosine phosphatase PTP1C as a B-cell antigen receptor-associated protein involved in the regulation of B-cell signaling. *J. Exp. Med.* **181**, 2077-2084.
- Partanen, J., Makela, T.P., Alitalo, R., Lehvaslaiho, H. and Alitalo, K. (1990) Putative tyrosine kinases expressed in K-562 human leukemia-cells. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8913-8917.
- Pascal, S.M., Singer, A.U., Gish, G., Yamazaki, T., Shoelson, S.E., Pawson, T., Kay, L.E. and Formankay, J.D. (1994) Nuclear-magnetic-resonance structure of an SH2 domain of phospholipase c- $\gamma$ 1 complexed with a high-affinity binding peptide. *Cell* **77**, 461-472.
- Pawson, T. and Gish, G.D. (1992) SH2 and SH3 domains - from structure to function. *Cell* **71**, 359-362.
- Pawson, T. (1995) Protein modules and signalling networks. *Nature* **373**, 573-580.
- Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J. and Sturgill, T.W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein-kinase (Map kinase). *EMBO J.* **10**, 885-892.
- Pei, D., Lorenz, U., Klingmuller, U., Neel, B.G. and Walsh, C.T. (1994) Intramolecular regulation of protein tyrosine phosphatase SH-PTP1: A new function for Src homology 2 domains. *Biochemistry* **33**, 15483-15493.

- Pei, D., Wang, J. and Walsh, C.T. (1996) Differential functions of the two Src homology 2 domains in protein tyrosine phosphatase SH-PTP1. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1141-1145.
- Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T. and Pelicci, P.G. (1992) A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**, 93-104.
- Perkins, L.A., Larsen, I. and Perrimon, N. (1992) Corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. *Cell* **70**, 225-236.
- Pluskey, S., Wandless, T.J., Walsh, C.T. and Shoelson, S.E. (1995) Potent stimulation of SH-PTP2 phosphatase activity by simultaneous occupancy of both SH2 domains. *J. Biol. Chem.* **270**, 2897-2900.
- Plutzky, J., Neel, B.G. and Rosenberg, R.D. (1992) Isolation of a src homology 2-containing tyrosine phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1123-1127.
- Polakis, P. and McCormick, F. (1993) Structural requirements for the interaction of p21ras with gap, exchange factors, and its biological effector target. *J. Biol. Chem.* **268**, 9157-9160.
- Pratt, J.C., Weiss, M., Sieff, C.A., Shoelson, S.E., Burakoff, S.J. and Ravichandran, K.S. (1996) Evidence for a physical association between the Shc-PTB domain and the beta(c) chain of the granulocyte-macrophage colony-stimulating factor receptor. *J. Biol. Chem.* **271**, 12137-12140.
- Pronk, G.J., McGlade, J., Pelicci, G., Pawson, T. and Bos, J.L. (1993) Insulin-induced phosphorylation of the 46-kda and 52-kda shc proteins. *J. Biol. Chem.* **268**, 5748-5753.
- Qu, C.K., Shi, Z.Q., Shen, R., Tsai, F.Y., Orkin, S.H. and Feng, G.S. (1997) A deletion mutation in the SH2-N domain of SHP-2 severely suppresses hematopoietic cell development. *Mol. Cell. Biol.* **17**, 5499-5507.
- Quelle, F.W., Sato, N., Witthuhn, B.S., Inhorn, R.C., Eder, M., Miyajima, A., Griffin, J.D. and Ihle, J.N. (1994) Jak2 associates with the beta(C) chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol. Cell. Biol.* **14**, 4335-4341.
- Quilliam, L.A., Huff, S.Y., Rabun, K.M., Wei, W., Park, W., Broek, D. and Der, C.J. (1994) Membrane-targeting potentiates guanine-nucleotide exchange factor cdc25 and sos1 activation of ras transforming activity. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8512-8516.
- Rane, S.G. and Reddy, E.P. (1994) Jak3-a novel Jak kinase associated with terminal differentiation of hematopoietic cells. *Oncogene* **9**, 2415-2423.

- Rao, P. and Mufson, R.A. (1995) A membrane proximal domain of the human interleukin-3 receptor beta(C) subunit that signals DNA-synthesis in NIH 3T3 cells specifically binds a complex of src and janus family tyrosine kinases and phosphatidylinositol 3-kinase. *J. Biol. Chem.* **270**, 6886-6893.
- Rapp, U.R., Goldsborough, M.D., Mark, G.E., Bonner, T.I., Groffen, J., Reynolds, F.H. and Stephenson, J.R. (1983) Structure and biological-activity of v-Raf, a unique oncogene transduced by a retrovirus. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4218-4222.
- Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993) Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259**, 1157-1161.
- Ricketts, W.A., Rose, D.W., Shoelson, S. and Olefsky, J.M. (1996) Functional roles of the Shc phosphotyrosine binding and Src homology- 2 domains in insulin and epidermal growth-factor signaling. *J. Biol. Chem.* **271**, 26165-26169.
- Roche, S., McGlade, J., Jones, M., Gish, G.D., Pawson, T. and Courtneidge, S.A. (1996) Requirement of phospholipase C-gamma, the tyrosine phosphatase Syp and the adapter proteins Shc and Nck for PDGF-induced DNA-synthesis - evidence for the existence of ras-dependent and ras-independent pathways. *EMBO J.* **15**, 4940-4948.
- Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of ras. *Nature* **370**, 527-532.
- Rodriguez-Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D. and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* **15**, 2442-2451.
- Roehm, N.W., Rodgers, G.H., Hatfield, S.M., and Glasebrook, A.L. (1991) An improved colormetric assay for cell proliferation and viability utilising the tetrazolium salt XTT. *J. Immunol. Meth.* **142**, 257-265.
- Rogge, R.D., Karlovich, C.A. and Banerjee, U. (1991) Genetic dissection of a neurodevelopmental pathway - son of sevenless functions downstream of the sevenless and EGF receptor tyrosine kinases. *Cell* **64**, 39-48.
- Rousse, J., Cohen, P., Trigon, S., Maorange, M., Alonsollamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A.R. (1994) A novel kinase cascade triggered by stress and heat-shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat-shock proteins. *Cell* **78**, 1027-1037.
- Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J. and Pawson, T. (1992) Association of the shc and grb2/sem5 SH2-containing proteins is implicated in activation of the ras pathway by tyrosine kinases. *Nature* **360**, 689-692.

- Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian grb2 couple the EGF receptor to the ras activator msos1. *Nature* **363**, 83-85.
- Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K. and Cohen, S. (1993) Epidermal growth-factor stimulates the tyrosine phosphorylation of shc in the mouse. *J. Biol. Chem.* **268**, 7610-7612.
- Sadowski, I., Stone, J.C. and Pawson, T. (1986) A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of fujinami sarcoma-virus p130gag-fps. *Mol. Cell. Biol.* **6**, 4396-4408.
- Saito, Y., Gomez, N., Campbell, D.G., Ashworth, A., Marshall, C.J. and Cohen, P. (1994) The threonine residues in MAP kinase kinase-1 phosphorylated by MAP kinase in-vitro are also phosphorylated in nerve growth factor-stimulated rat pheochromocytoma (PC12) cells. *FEBS Lett.* **341**, 119-124.
- Sakaguchi, K., Okabayashi, Y., Kido, Y., Kimura, S., Matsumura, Y., Inushima, K. and Kasuga, M. (1998) Shc phosphotyrosine-binding domain dominantly interacts with epidermal growth factor receptors and mediates Ras activation in intact cells. *Molecular Endocrinology* **12**, 536-543.
- Sakamaki, K., Miyajima, I., Kitamura, T. and Miyajima, A. (1992) Critical cytoplasmic domains of the common beta-subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. *EMBO J.* **11**, 3541-3549.
- Salcini, A.E., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T. and Pelicci, P.G. (1994) Formation of shc-grb2 complexes is necessary to induce neoplastic transformation by overexpression of shc proteins. *Oncogene* **9**, 2827-2836.
- Samuels, M.L., Weber, M.J., Bishop, J.M. and McMahon, M. (1993) Conditional transformation of cells and rapid activation of the mitogen-activated protein-kinase cascade by an estradiol-dependent human Raf-1 protein-kinase. *Mol. Cell. Biol.* **13**, 6241-6252.
- Sasaoka, T., Ishihara, H., Sawa, T., Ishiki, M., Morioka, H., Imamura, T., Usui, I., Takata, Y. and Kobayashi, M. (1996) Functional importance of amino-terminal domain of Shc for interaction with insulin and epidermal growth factor receptors in phosphorylation-independent manner. *J. Biol. Chem.* **271**, 20082-20087.
- Sato, N., Sakamaki, K., Terada, N., Arai, K. and Miyajima, A. (1993) Signal-transduction by the high-affinity GM-CSF receptor - 2 distinct cytoplasmic regions of the common beta-subunit responsible for different signaling. *EMBO J.* **12**, 4181-4189.
- Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T. and Kaziro, Y. (1990a) Accumulation of p21ras.GTP in response to stimulation with epidermal growth-factor

- and oncogene products with tyrosine kinase-activity. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7926-7929.
- Satoh, T., Endo, M., Nakafuku, M., Nakamura, S. and Kaziro, Y. (1990b) Platelet-derived growth-factor stimulates formation of active p21ras.Gtp complex in swiss mouse 3T3-cells. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5993-5997.
- Satoh, T., Nakafuku, M., Miyajima, A. and Kaziro, Y. (1991) Involvement of ras p21 protein in signal-transduction pathways from interleukin-2, interleukin-3, and granulocyte macrophage colony- stimulating factor, but not from interleukin-4. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3314-3318.
- Satoh, T., Nakafuku, M. and Kaziro, Y. (1992) Function of ras as a molecular switch in signal transduction. *J. Biol. Chem.* **267**, 24149-24152.
- Saxton, T.M., Henkemeyer, M., Gasca, S., Shen, R., Rossi, D.J., Shalaby, F., Feng, G.S. and Pawson, T. (1997) Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J.* **16**, 2352-2364.
- Schaeffer, H.J., Catling, A.D., Eblen, S.T., Collier, L.S., Krauss, A. and Weber, M.J. (1998) MP1: A MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281**, 1668-1671.
- Scheid, M.P., Lauener, R.W., and Duronio, V. (1995) Role of phosphatidylinositol 3-OH-kinase activity in the inhibition of apoptosis in haemopoietic cells: phosphatidylinositol 3-OH-kinase inhibitors reveal a difference in signalling between interleukin-3 and granulocyte-macrophage colony stimulating factor. *Biochem J.* **312**, 159-162.
- Scheid, M.P. and Duronio, V. (1996) Phosphatidylinositol 3-OH kinase activity is not required for activation of mitogen-activated protein kinase by cytokines. *J. Biol. Chem.* **271**, 18134-18139.
- Schindler, C. and Darnell, J.E. (1995) Transcriptional responses to polypeptide ligands - the jak-stat pathway. *Ann. Rev. Biochem.* **64**, 621-651.
- Schrader, J.W. and Nossal, G.J.V. (1980) Strategies for the analysis of accessory-cell function: the in vitro cloning and characterization of the P cell. *Immunol.Rev.* **53**, 61-85.
- Schrader, J.W. (1981) The in vitro production and cloning of the P-cell, a bone marrow-derived null-cell that expresses H-2 and IA-antigens, has mast cell-like granules, and is regulated by a factor released by activated T-cells. *J. Immunol.* **126**, 452-458.
- Schrader, J.W. (1986) The panspecific hemopoietin of activated T lymphocytes (interleukin-3). *Ann.Rev.Immunol.* **4**, 205-230.

- Sharma, P.M., Egawa, K., Huang, Y., Martin, J.L., Huvar, I., Boss, G.R. and Olefsky, J.M. (1998) Inhibition of phosphatidylinositol 3-kinase activity by adenovirus-mediated gene transfer and its effect on insulin action. *J. Biol. Chem.* **273**, 18528-18537.
- Shen, S.H., Bastien, L., Posner, B.I. and Chretien, P. (1991) A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature* **352**, 736-739.
- Shi, Z.Q. (1998) The SHP-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH2-terminal mitogen-activated protein kinases. *J. Biol. Chem.* **273**, 4902-4908.
- Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., Kakata, T., Feramisco, J., Stavnezer, E., Fogh, J. and Wigler, M.H. (1983) 3 human transforming genes are related to the viral Ras oncogenes. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2112-2116.
- Shultz, L.D., Schweitzer, P.A., Rajan, T.V., Yi, T.L., Ihle, J.N., Matthews, R.J., Thomas, M.L. and Beier, D.R. (1993) Mutations at the murine moth-eaten locus are within the hematopoietic-cell protein-tyrosine-phosphatase (Hcph) Gene. *Cell* **73**, 1445-1454.
- Sideras, P. and Palacios, R. (1987) Bone-marrow pro-lymphocyte-T and pro-lymphocyte-B clones express functional receptors for interleukin (IL) 3 and IL-4/BSF-1 and nonfunctional receptors for IL-2. *Eur. J. Immunol.* **17**, 217-221.
- Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Cleveland, J.L., Yi, T. and Ihle, J.N. (1993) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. **90**, 8429-8433.
- Simon, M.A., Bowtell, D.D.L., Dodson, G.S., Lavery, T.R. and Rubin, G.M. (1991) Ras1 and a putative guanine-nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* **67**, 701-716.
- Simon, M.A., Dodson, G.S. and Rubin, G.M. (1993) An SH3-SH2-SH3 protein is required for p21(Ras1) activation and binds to sevenless and sos proteins invitro. *Cell* **73**, 169-177.
- Skolnik, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, M.J., Backer, J.M., Ullrich, A., White, M.F. and Schlessinger, J. (1993) The SH2 SH3 domain-containing protein grb2 interacts with tyrosine-phosphorylated IRS1 and shc - implications for insulin control of ras signaling. *EMBO J.* **12**, 1929-1936.
- Smith, M.R., Degudicibus, S.J. and Stacey, D.W. (1986) Requirement for c-ras proteins during viral oncogene transformation. *Nature* **320**, 540-543.
- Soler, C., Alvarez, C.V., Beguinot, L. and Carpenter, G. (1994) Potent shc tyrosine phosphorylation by epidermal growth-factor at low receptor density or in the absence of receptor autophosphorylation sites. *Oncogene* **9**, 2207-2215.

- Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schaffhausen, B. and Cantley, L.C. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-778.
- Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R.A. and Cantley, L.C. (1994) Specific motifs recognized by the SH2 domains of CSK, 3BP2, fps/fes, Grb-2, Hcp, Shc, Syk, and Vav. *Mol. Cell. Biol.* **14**, 2777-2785.
- Songyang, Z., Margolis, B., Chaudhuri, M., Shoelson, S.E. and Cantley, L.C. (1995) The phosphotyrosine interaction domain of SHC recognizes tyrosine-phosphorylated NPXY motif. *J. Biol. Chem.* **270**, 14863-14866.
- Stanton, V.P., Nichols, D.W., Laudano, A.P. and Cooper, G.M. (1989) Definition of the human raf amino-terminal regulatory region by deletion mutagenesis. *Mol. Cell. Biol.* **9**, 639-647.
- Stephens, L.R., Jackson, T.R. and Hawkins, P.T. (1993) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate - a new intracellular signaling system. *Biochimica Et Biophysica Acta* **1179**, 27-75.
- Stephens, R.M., Loeb, D.M., Copeland, T.D., Pawson, T., Greene, L.A. and Kaplan, D.R. (1994) Trk receptors use redundant signal-transduction pathways involving Shc and PLC-gamma-1 to mediate responses. *Neuron* **12**, 691-705.
- Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. (1994) Activation of raf as a result of recruitment to the plasma-membrane. *Science* **264**, 1463-1467.
- Sturgill, T.W., Ray, L.B., Erikson, E. and Maller, J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal-protein S6 kinase-II. *Nature* **334**, 715-718.
- Sugimoto, S., Lechleider, R.J., Shoelson, S.E., Neel, B.G. and Walsh, C.T. (1993) Expression, purification, and characterization of SH2-containing protein tyrosine phosphatase, SH-PTP2. *J. Biol. Chem.* **268**, 22771-22776.
- Sugimoto, S., Wandless, T.J., Shoelson, S.E., Neel, B.G. and Walsh, C.T. (1994) Activation of the SH2-containing protein-tyrosine-phosphatase, SH-PTP2, by phosphotyrosine-containing peptides derived from insulin- receptor substrate-1. *J. Biol. Chem.* **269**, 13614-13622.
- Tang, T.L., Freeman, R.M., Oreilly, A.M., Neel, B.G. and Sokol, S.Y. (1995) The SH2-containing protein-tyrosine-phosphatase SH-PTP2 is required upstream of map kinase for early xenopus development. *Cell* **80**, 473-483.



- Tanner, J.W., Chen, W., Young, R.L., Longmore, G.D. and Shaw, A.S. (1995) The conserved box-1 motif of cytokine receptors is required for association with jak kinases. *J. Biol. Chem.* **270**, 6523-6530.
- Tauchi, T., Feng, G.S., Marshall, M.S., Shen, R., Mantel, C., Pawson, T. and Broxmeyer, H.E. (1994) The ubiquitously expressed Syp phosphatase interacts with c-kit and Grb2 in hematopoietic cells. *J. Biol. Chem.* **269**, 25206-25211.
- Tauchi, T., Feng, G.S., Shen, R., Hoatlin, M., Bagby, G., Kabat, D., Lu, L. and Broxmeyer, H.E. (1995) Involvement of SH2-containing phosphotyrosine phosphatase Syp in erythropoietin receptor signal transduction pathways. *J. Biol. Chem.* **270**, 5631-5635.
- Tauchi, T., Damen, J.E., Toyama, K., Feng, G.S., Broxmeyer, H.E. and Krystal, G. (1996) Tyrosine 425 within the activated erythropoietin receptor binds Syp, reduces the erythropoietin required for Syp tyrosine phosphorylation, and promotes mitogenesis. *Blood* **87**, 4495-4501.
- Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wasserman, D.A. and Rubin, G.M. (1995) Ksr, a novel protein-kinase required for ras signal-transduction. *Cell* **83**, 879-888.
- Thomas, D. and Bradshaw, R.A. (1997) Differential utilization of ShcA tyrosine residues and functional domains in the transduction of epidermal growth factor-induced mitogen-activated protein kinase activation in 293T cells and nerve growth factor-induced neurite outgrowth in PC12 cells - Identification of a new Grb2-Sos1 binding site. *J. Biol. Chem.* **272**, 22293-22299.
- Tonks, N.K. and Neel, B.G. (1996) From form to function: Signaling by protein tyrosine phosphatases. *Cell* **87**, 365-368.
- Trahey, M. and McCormick, F. (1987) A cytoplasmic protein stimulates normal n-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238**, 542-545.
- Trub, T., Choi, W.E., Wolf, G., Ottinger, E., Chen, Y.J., Weiss, M. and Shoelson, S.E. (1995) Specificity of the PTB domain of Shc for  $\beta$  turn-forming pentapeptide motifs amino-terminal to phosphotyrosine. *J. Biol. Chem.* **270**, 18205-18208.
- Tsui, H.W., Siminovitch, K.A., Desouza, L. and Tsui, F.W.L. (1993) Moth-eaten and viable moth-eaten mice have mutations in the hematopoietic-cell phosphatase gene. *Nature Genetics* **4**, 124-129.
- Tzivion, G., Luo, Z.J. and Avruch, J. (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394**, 88-92.
- Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase-activity. *Cell* **61**, 203-212.

- Vanaelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993) Complex-formation between ras and raf and other protein-kinases. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6213-6217.
- Van der Geer, P., Wiley, S., Lai, V.K.M., Olivier, J.P., Gish, G.D., Stephens, R., Kaplan, D., Shoelson, S. and Pawson, T. (1995) A conserved amino-terminal shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Curr. Biol.* **5**, 404-412.
- Vanhaesebroeck, B., Leeyers, S.J., Panayotou, G. and Waterfield, M.D. (1997a) Phosphoinositide 3-kinases: A conserved family of signal transducers. *Trend. Biochem. Sci.* **22**, 267-272.
- Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M.D. (1997b) p110 delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4330-4335.
- Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian ras interacts directly with the serine threonine kinase raf. *Cell* **74**, 205-214.
- Von Willebrand, M., Jascur, T., BonnefoyBerard, N., Yano, H., Altman, A., Matsuda, Y. and Mustelin, T. (1996) Inhibition of phosphatidylinositol 3-kinase blocks T cell antigen receptor CD3-induced activation of the mitogen-activated kinase Erk2. *Eur. J. Biochem.* **235**, 828-835.
- Waksman, G., Kominos, D., Robertson, S.C., Pant, N., Baltimore, D., Birge, R.B., Cowburn, D., Hanafusa, H., Mayer, B.J., Overduin, M., Resh, M.D., Rios, C.B., Silverman, L. and Kuriyan, J. (1992) Crystal-structure of the phosphotyrosine recognition domain SH2 of v- src complexed with tyrosine-phosphorylated peptides. *Nature* **358**, 646-653.
- Waksman, G., Shoelson, S.E., Pant, N., Cowburn, D. and Kuriyan, J. (1993) Binding of a high-affinity phosphotyrosyl peptide to the src SH2 domain - crystal-structures of the complexed and peptide-free forms. *Cell* **72**, 779-790.
- Watanabe, S., Itoh, T. and Arai, K. (1996) JAK2 is essential for activation of c-fos and c-myc promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells. *J. Biol. Chem.* **271**, 12681-12686.
- Welham, M.J., Duronio, V., Sanghera, J.S., Pelech, S.L. and Schrader, J.W. (1992) Multiple hemopoietic growth factors stimulate activation of mitogen- activated protein kinase family members. *J. Immunol.* **149**, 1683-1693.
- Welham, M.J., Duronio, V., Leslie, K.B., Bowtell, D. and Schrader, J.W. (1994a) Multiple hemopoietins, with the exception of interleukin-4, induce modification of Shc and mSos1, but not their translocation. *J. Biol. Chem.* **269**, 21165-21176.

- Welham, M.J., Dechert, U., Leslie, K.B., Jirik, F. and Schrader, J.W. (1994b) Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase. *J. Biol. Chem.* **269**, 23764-23768.
- Welsh, G.I., Foulstone, E.J., Young, S.W., Tavaré, J.M. and Proud, C.G. (1994) Wortmannin inhibits the effects of insulin and serum on the activities of glycogen-synthase kinase-3 and mitogen-activated protein-kinase. *Biochem. J.* **303**, 15-20.
- Weng, Q.P., Andrabi, K., Klippel, A., Kozlowski, M.T., Williams, L.T. and Avruch, J. (1995) Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase in-situ through site-specific p70 phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5744-5748.
- Whitmarsh, A.J. and Davis, R.J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.* **74**, 589-607.
- Wilks, A.F. (1989) 2 Putative protein-tyrosine kinases identified by application of the polymerase chain-reaction. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1603-1607.
- Williams, N.G., Paradis, H., Agarwall, S., Charest, D.L., Pelech, S.L. and Roberts, T.M. (1993) Raf-1 and p21(v-Ras) cooperate in the activation of mitogen-activated protein-kinase. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5772-5776.
- Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T.L., Tang, B., Miura, O. and Ihle, J.N. (1993) Jak2 associates with the erythropoietin receptor and is tyrosine-phosphorylated and activated following stimulation with erythropoietin. *Cell* **74**, 227-236.
- Wodnar-Filipowicz, A., Heusser, C.H. and Moroni, C. (1989) Production of the hematopoietic growth-factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. *Nature* **339**, 150-152.
- Wu, J., Harrison, J.K., Vincent, L.A., Haystead, T.A.J., Michel, H., Hunt, D.F., Lynch, K.R. and Sturgill, T.W. (1993a) Molecular cloning of a protein-tyrosine/threonine kinase activating mitogen-activated protein (MAP) kinase: MAP kinase kinase. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 173-177.
- Wu, J., Harrison, J.K., Dent, P., Lynch, K.R., Weber, M.J. and Sturgill, T.W. (1993b) Identification and characterization of a new mammalian mitogen-activated protein kinase kinase, MKK2. *Mol. Cell. Biol.* **8**, 4539-4548.
- Xiao, S., Rose, D.W., Sasaoka, T., Maegawa, H., Burke, T., Roller, P.P., Shoelson, S.E. and Olefsky, J.M. (1994) Syp (SH-PTP2) is a positive mediator of growth factor-stimulated mitogenic signal transduction. *J. Biol. Chem.* **269**, 21244-21248.
- Xu, G.F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R. and Tamanoi, F. (1990) The catalytic domain of the neurofibromatosis type-1 gene-

- product stimulates ras GTPase and complements ira mutants of *saccharomyces-cerevisiae*. *Cell* **63**, 835-841.
- Yamauchi, K., Milarski, K.L., Saltiel, A.R. and Pessin, J.E. (1995) Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 664-668.
- Yang, Y.-C., Kovacic, S., Kriz, R., Wolf, S., Clark, S., Wellems, T., Nienhuis, A. and Epstein, N. (1988) The human genes for GM-CSF and IL-3 are closely linked in tandem on chromosome-5. *Blood* **71**, 961.
- Yao, R.J. and Cooper, G.M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth-factor. *Science* **267**, 2003-2006.
- Yi, T., Mui, A.L.F., Krystal, G. and Ihle, J.N. (1993) Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. *Mol. Cell. Biol.* **13**, 7577-7586.
- Yi, T., Zhang, J., Miura, O. and Ihle, J.N. (1995) Hematopoietic cell phosphatase associates with erythropoietin (Epo) receptor after Epo-induced receptor tyrosine phosphorylation: Identification of potential binding sites. *Blood* **85**, 87-95.
- Yi, T.L., Cleveland, J.L. and Ihle, J.N. (1992) Protein tyrosine phosphatase containing SH2 domains - characterization, preferential expression in hematopoietic-cells, and localization to human-chromosome 12p12-p13. *Mol. Cell. Biol.* **12**, 836-846.
- Yi, T.L. and Ihle, J.N. (1993) Association of hematopoietic-cell phosphatase with c-kit after stimulation with c-kit ligand. *Mol. Cell. Biol.* **13**, 3350-3358.
- Yin, T., Shen, R., Feng, G.S. and Yang, Y.C. (1997) Molecular characterization of specific interactions between SHP-2 phosphatase and JAK tyrosine kinases. *J. Biol. Chem.* **272**, 1032-1037.
- Yokota, T., Lee, F., Rennick, D., Hall, C., Arai, N., Mosmann, T., Nabel, G., Cantor, H. and Arai, K.I. (1984) Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1070-1074.
- Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C.H. and Claessonwelsch, L. (1994) Direct interaction between shc and the platelet-derived growth-factor beta-receptor. *J. Biol. Chem.* **269**, 15337-15343.
- Yonehara, S., Ishii, A., Yonehara, M., Koyasu, S., Miyajima, A., Schreurs, J., Arai, K. and Yahara, I. (1990) Identification of a cell-surface 105 kd protein (Aic-2 antigen) which binds interleukin-3. *International Immunology* **2**, 143-150.

- Yu, W., Fantl, W.J., Harrowe, G. and Williams, L.T. (1997) Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK. *Current Biol.* **8**, 56-64.
- Zha, J.P., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-628.
- Zhang, X.F., Settleman, J., Kyriakis, J.M., Takeuchisuzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. and Avruch, J. (1993) Normal and oncogenic p21(Ras) Proteins bind to the amino-terminal regulatory domain of c-raf-1. *Nature* **364**, 308-313.
- Zhao, Y.M., Wagner, F., Frank, S.J. and Kraft, A.S. (1995) The amino-terminal portion of the jak2 protein-kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor-receptor beta(C) Chain. *J. Biol. Chem.* **270**, 13814-13818.
- Zhao, Z.Z., Larocque, R., Ho, W.T., Fischer, E.H. and Shen, S.H. (1994) Purification and characterization of PTP2C, a widely distributed protein-tyrosine-phosphatase containing 2 SH2 domains. *J. Biol. Chem.* **269**, 8780-8785.
- Zheng, C.F. and Guan, K.L. (1993) Cloning and characterization of 2 distinct human extracellular signal-regulated kinase activator kinases, mek1 and mek2. *J. Biol. Chem.* **268**, 11435-11439.
- Zhou, M.M., Ravichandran, K.S., Olejniczak, E.T., Petros, A.M., Meadows, R.P., Sattler, M., Harlan, J.E., Wade, W.S., Burakoff, S.J. and Fesik, S.W. (1995a) Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature* **378**, 584-592.
- Zhou, M.M., Harlan, J.E., Wade, W.S., Crosby, S., Ravichandran, K.S., Burakoff, S.J. and Fesik, S.W. (1995b) Binding affinities of tyrosine-phosphorylated peptides to the COOH-terminal SH2 and NH2-terminal phosphotyrosine binding domains of Shc. *J. Biol. Chem.* **270**, 31119-31123.
- Ziegler, S.F., Bird, T.A., Morella, K.K., Mosley, B., Gearing, D.P. and Baumann, H. (1993) Distinct regions of the human granulocyte-colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. *Mol. Cell. Biol.* **13**, 2384-2390.
- Ziltener, H.J., Destgroth, B.F., Leslie, K.B. and Schrader, J.W. (1988) Multiple glycosylated forms of T-cell-derived interleukin-3 (IL-3)- heterogeneity of IL-3 from physiological and non-physiological sources. *J. Biol. Chem.* **263**, 14511-14517.
- Ziltener, H.J., Clark-Lewis, I., Jones, A.T. and Dy, M. (1994) Carbohydrate does not modulate the in-vivo effects of injected interleukin-3. *Exp. Hematol.* **22**, 1070-1075.

- Zurawski, S.M., Vega, F. Jr., Huyghe, B., and Zurawski, G. (1993). Receptors for interleukin-13 and interleukin-4 are complex and share a novel compenet that functions in signal transduction. *EMBO J.* **12**, 2663-2670.

**List of Publications**

- Bone, H.**, Dechert, U., Jirik, F., Schrader, J.W. and Welham, M.J. SHP1 and SHP2 Protein-tyrosine Phosphatases Associate with  $\beta c$  after Interleukin-3-induced Receptor Tyrosine Phosphorylation. *J. Biol. Chem.* **272**, 14470-14476.
- Bone, H.** and Welham, M.J. (1999) Association between  $\beta c$  and Shc After Interleukin-3-Induced Tyrosine Phosphorylation is mediated by both the SH2 and PTB domains of Shc. Submitted.
- Craddock, B.L., **Bone, H.**, and Welham, M.J. (1999) Requirement of Shc and Phosphoinositol-3 kinase in Interleukin-3 Regulation of the Mitogen-Activated Protein Kinase Pathway. In preparation.